

THE DEVELOPMENT AND APPLICATION OF
METHODS FOR USING 'AGROBACTERIUM' SPP. AS
DNA VECTORS IN SOFT FRUIT PLANTS

Julie Graham

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**THE DEVELOPMENT AND APPLICATION OF METHODS FOR USING
Agrobacterium spp. AS DNA VECTORS IN SOFT FRUIT PLANTS.**

by Julie Graham.

A thesis presented for the degree of Doctor of
Philosophy at the University of St. Andrews.

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Abstract.

Improvements in soft fruit cultivars have been achieved by the selection of superior genotypes through plant breeding. Limitations imposed by plant breeding, led to work being initiated into the development of a gene vector technique for soft fruit, to permit improvement without altering their overall genetic makeup.

Little research has been carried out in this area with perennial crops, cultivars of which are highly heterozygous, their genetic structure being maintained by vegetative propagation.

Probably the most successful method of transformation available involves the use of the soil bacterium Agrobacterium. This bacterium is manipulated to contain the gene of interest, and used to infect small explants of the genotype which, can result in gene transfer to some cells of the explant. These transformed cells are induced to regenerate whole plants, which are assessed to select those containing the gene of interest.

A transformation system suitable for use in three soft fruit species (Rubus, Ribes and Fragaria spp.) was developed. Initially these species and Vaccinium spp. were inoculated with a range of wild type Agrobacterium isolates to demonstrate the ability of the isolates to infect. All but one isolate was capable of effecting gene transfer (to some degree) into the plants, which was demonstrated by the production of disease symptoms.

Whole plant inoculation was not useful for Agrobacterium transformation, as only the cells at the

site of infection contained the foreign DNA.

Regeneration techniques were developed for each species, so that the infected cell(s) would regenerate to form whole plants containing the foreign DNA in every cell.

With Rubus spp., both leaf disc and internodal segment explants were induced to regenerate whole plants. Generally a larger number of internodal segments than leaf discs regenerated, though the productivity of plantlets from a leaf disc was greater. Cultivars differed in their ability to regenerate.

For blackcurrant, an internodal segment system was developed, and for strawberry, leaf disc regeneration was achieved. In Vaccinium spp. regeneration occurred using leaf material.

Using these regeneration techniques, transformation experiments were initiated using both the Neomycin phosphotransferase II (NPT II) and the Beta-Glucuronidase (GUS) marker genes. Once putative transformants were selected on kanamycin medium, dot blot, fluorometric, histochemical, and Southern hybridisation assays were carried out. The GUS gene proved especially useful in the identification of transformants, providing a reliable, simple, quick and inexpensive marker gene.

The development of transformation systems in soft fruit species has led to experiments being initiated for plant improvement, by the insertion of specific genes, notably genes conferring virus and disease resistance on Rubus and strawberry plants by Agrobacterium.

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Declaration

I, Julie Graham, declare that this thesis is my own work, and that it has not been submitted for any other degree.

Julie Graham.

St, Andrews, November 1990.

Supervisors Certificate

We certify that Julie Graham has conducted the appropriate period of research under our direction, that she has fulfilled the conditions of Ordinance General No. 12 and the Resolution of the University Court 1967 No. 1, and that she is qualified to submit this thesis for the degree of Doctor of Philosophy.

Prof. N.L. Innes

Dr. R.J. Abbott

St. Andrews November 1990

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Chapter 1.

The development of a gene vector system based on Agrobacterium for the introduction of foreign genes into soft fruit species.

The development of a gene vector system based on Agrobacterium for the introduction of foreign genes into soft fruit species.

In the improvement of cultivated plant species, conventional horticulture and agriculture have largely relied on plant breeding to select for more desirable characteristics. Although this has been highly successful, there are a number of drawbacks associated with the process, including the time scale involved, the limited ability to breed in a specific character (i.e. the desired character must exist within the species or perhaps a closely related species) and the co-transfer of undesirable characteristics.

Genetic engineering offers a method of overcoming these limitations, as well as providing a new tool which can be used in other areas of plant biotechnology such as gene isolation.

The Scottish Crop Research Institute, situated in the east of Scotland, is in the centre of a major soft fruit growing area. The Soft Fruit Genetics Department has been conducting plant breeding experiments for over 20 years. This plant breeding work has been very successful leading to the production of new cultivars such as the recently released black currant cvs. Ben Alder and Ben Tirran, the strawberry cv. Rapella, the blackberry cv. Loch Ness and red raspberry cvs. Glen Lyon and Glen Garry. However a number of drawbacks in the breeding programme and the ongoing research into the development

of transformation systems in tobacco by other researchers, led to the initiation of a new research aspect in the department concerned generally with "plant biotechnology" and in the first instance Agrobacterium transformation.

Little research has been carried out in this area with perennial crops such as soft fruit. Cultivars of these crops are highly heterozygous and their genotypic structure is maintained by vegetative propagation. A technique which would permit improvement without altering their overall genetic makeup would therefore be extremely valuable.

This thesis is concerned with the development of such a transformation system in order that potential improvements may be made in soft fruit species. This chapter mainly describes Agrobacterium biology, leading to the development of vectors based on the natural properties of the bacterium and its ability to transfer genes into plant tissues. A very brief introduction is given to the biology of the four soft fruit species used in the research reported in this thesis before Agrobacterium is described.

Crop Biology

Approximately 104,900 tonnes of soft fruit are produced annually in the U.K. with a value of £98,904,000 (Anon. 1988). This is made up of raspberries, blackberries and hybrid berries (Rubus

spp.), strawberries (Fragaria spp.), blackcurrants (Ribes spp.) and blueberries (Vaccinium spp.)

Rubus species.

Rubus is a diverse genus being divided into 12 subgenera, only 3 of which contain domesticated species of importance. Those of importance include subgenus Eubatus, the blackberries and dewberries which represent a very large number of species; Idaeobatus, the raspberries, of which there are 350 species; and Cylactis, close to Idaeobatus, containing 14 species including the arctic raspberry R. arcticus L., (Jennings 1988).

Rubus subgen. Idaeobatus is distributed principally in Asia but also East and South Africa, Europe and North America. In contrast, subgen. Eubatus is mainly distributed in South America, Europe and North America (Jennings 1988). The members of subgenus Idaeobatus sp. are distinguished by the ability of their mature fruits to separate from the receptacle. The subgenus is particularly well represented in the northern hemisphere.

Generally the canes grow one year and fruit the next but there are also primocane varieties which fruit in the first year. The biennial growth cycle of raspberry and blackberry stems begins when a bud from below soil level develops and elongation of the internodes carries the growing point, protected by leaf scales, to the soil

surface. At the surface, leaves expand to form a tight rosette around the growing point. Elongation of the shoot starts in spring and continues until autumn, by which time the shoot will have attained a height of 2 to 3 m.

In red raspberries (R. idaeus L.), shortening days and falling temperatures in late summer cause shoot elongation to cease and dormancy to set in. This is a gradual process extending over several weeks and once a stage of complete dormancy is reached it is not readily reversible. Black raspberries (R. occidentalis L.) or purple raspberries (hybrids between red and black raspberries) and most blackberries differ from red raspberries both in time when dormancy begins and intensity of dormancy attained. In these fruits, growth continues well into autumn. The initiation of flower buds usually starts at the same time as the canes begin to acquire dormancy.

In the spring of the second year, vegetative primocanes become fruiting canes. The fruit is composed of a large number of one-seeded drupelets which are set together on a small conical core (Jennings 1988).

In Scotland large quantities of red raspberries (12,320 MT approx.)(Anon. 1988) are grown for processing. Early varieties are generally grown so that the picking season coincides with the school holidays. Of the 2200 ha grown in Scotland most occur in Tayside and central Scotland. In England around 1600 ha of raspberries are grown as a basket fruit. Generally later

varieties are grown whose production does not clash with strawberries (Gordon et al 1990).

In Scotland at present the dominant cultivars are Glen Clova and Malling Jewel. The removal from the market of the dessicant herbicide dinoseb, which managed the vigorous cane growth of Glen Clova, has led to the planting of new less vigorous cultivars e.g. Glen Moy and Glen Prosen. The mid season variety is Malling Delight followed by later season Norfolk Giant, Malling Admiral and Malling Leo.

Raspberry plantations if well managed last for many years. Generally they are tolerant to a wide range of soil conditions and do best where texture is such that water drainage is not impeded but moisture is retained.

There are a number of objectives in the breeding programme among which are good vigour, high productivity, increased hardiness, large fruit size, high fruit quality, strong primocane formation and erect sturdy canes which require no trellising. Fruit characteristics of importance include large fruit size, firmness (for handling) and good quality fruits with small bright coloured seeds (for jam). Breeding for resistance to pests is very important as a number of pests seriously damage Rubus crops either annually or occasionally.

Fragaria species.

Eleven wild species of Fragaria occur over four naturally occurring chromosome groups, diploid, tetraploid, hexaploid and octoploid (Darrow 1966).

The cultivated strawberry ($2n=56$), is of recent origin resulting from a cross between two wild American octoploid species Fragaria virginiana Duch. and F. chiloensis (L) Duch. (Scott and Lawrence 1975). The strawberry has become widely consumed in the past 50 years or so, due to strawberry breeding providing cultivars which fruit abundantly under widely different environmental conditions. The plants are low growing, spreading with runners which root to produce new plants. The crown of the plant produces shoots which flower and fruit. The juicy edible fruit is an enlarged receptacle on the surface of which seeds are embedded.

In the early years of this century the strawberry was considered to be an easy crop to grow due to having fewer pests and diseases than other fruit crops. Unfortunately, however, pathogens soon appeared, leading to the decline of old cultivars which disappeared. Virus diseases were the main trouble, followed by other problems such as red core disease caused by the fungus Phytophthora fragariae. These problems were overcome by the use of certified stocks and after the second world war cultivars bred at research stations were introduced. Auchincruive Climax followed by Huxley were introduced, though the stock lost vigour and was replaced by Red

Gauntlet which is still grown today. Today in the U.K. the most significant cultivar is Cambridge Favourite.

Strawberry growth, yield and fruit quality are greatly influenced by the interaction of a wide range of environmental and plant factors such as photoperiod, temperature, disease resistance, tolerance to different soil conditions, winterhardiness, high temperature tolerance and inherent vigour. Thus plants of a particular cultivar may grow well and be satisfactory in one area but may be unsatisfactory in another area where environmental conditions are different.

Modern cultivars have hermaphrodite flowers and therefore must be emasculated for controlled cross-pollinations. The blossom is composed of many pistils each with its own style and stigma attached to a receptacle which on fertilisation of the pistils develops into a fleshy fruit. Strawberry selections and cultivars are propagated vegetatively by runner plants rather than by seed (Scott and Lawrence 1975).

In the U.K. around 6,467 ha of land are used for strawberries, producing 8.1 tonnes per hectare (52,382 MT per year)(Anon. 1988). Breeding for improved fruit characteristics such as size, symmetry, shape, flesh firmness, skin firmness, colour, gloss, flavour, ease of removing husk, vitamin content, acidity and resistance to rot are carried out as well as for improved plant characteristics including yield, vigour, fruiting habit, time of ripening, winterhardiness, blossom frost-hardiness, disease resistance and virus tolerance.

Vaccinium species.

Blueberries are species of plants belonging to the genus Vaccinium. They are fruit bearing perennial shrubs with widely varying growth habits. The highbush types have been the subject of much breeding and research in the United States and Canada and today's varieties are derived mostly from the North American species V. corymbosum L., V. australe Small and to a lesser extent, a lowbush species V. angustifolium Aiton. There is a high incidence of polyploidy in the genus ($x=12$) with many natural tetraploids and hexaploids.

The highbush blueberry generally grows to a height of 1-3 metres. Shoots emerge each year from almost anywhere on the bush, the most vigorous ones starting near the base. In their second year these shoots produce flower trusses from varying numbers of buds at their tips while, from a number of buds below these, lateral branches appear which bear flowers in the following year. After 2 or 3 years the fruiting units become weak and twiggy and frequently die out. In favourable conditions over three-quarters of the flowers produced by the highbush blueberry develop into fruit. The berries are blue-black, generally round and can grow to a maximum size of about 2 cm diameter. The blueberry grows best in moist acid soils which do not dry out or become waterlogged. The climatic requirements of regular rainfall, 160 growing days and 650-850 hours below 7°C during winter are met in most parts of Britain (Cormack 1979).

Domesticated entirely in the twentieth century, blueberry culture in Britain at this time is fairly free of insect pests, the only control measure necessary at the moment is an occasional spray against the tortrix moth.

Vaccinium breeding in the soft fruit genetics department at S.C.R.I. was initiated in 1987 when initial crosses were made to produce cultivars adapted to UK production with good fruit quality, climatic adaptation and disease resistance.

Ribes species.

All species and commercial cultivars are diploid with the basic chromosome number $x=8$. Polyploidy is unknown in the wild and in cultivation only 2 cases have been reported (Keep 1975). The European blackcurrant, R. nigrum L. has become domesticated within the last 400 years with the first published records of the species being in 17th century herbals (Roach 1985). Shoot primordia are laid down in buds on the current years growth and when growing conditions are satisfactory flowers are formed within the majority of buds on the new shoots. Pollination in blackcurrants is carried out by insects (Keep 1975). Each pollen grain which germinates sends out a pollen tube which grows down the style and into the ovary where it enters an ovule to effect fertilisation. Each time this happens, a seed is produced and the development of the enclosing fruitlet depends on it containing a sufficient number of viable

seeds. When the ovules have been fertilised, the developing fruitlet rapidly increases in size (Anon. 1973).

By the 19th Century, several cultivars were available including Baldwin, Black Naples and Black Grape all of which are still grown. Baldwin at around 150 years old, is still the leading cultivar in Britain. The majority of commercial varieties were developed from Ribes nigrum believed to be introduced into the U.K. from its native habitats in Asia, Russia and Central Europe during the 15th and 16th Century.

The fruit is valued today for its exceptionally high vitamin C content with 29000 MT produced in the U.K. each year on 3000ha land. The crop is estimated to have a value of 13.2 million pounds per annum (Brennan 1989). Breeding objectives in blackcurrant include bushes suited to mechanical harvest or easier and quicker to hand pick, spring frost resistance or avoidance and better cropping capacity. Spraying is required against a number of pests and an ongoing breeding programme for resistance is in process. Breeding for juice quality is the main objective in the programme due to the market for blackcurrant juice in the U.K.

Agrobacterium Biology and the Development of Vector Systems.

With the increasing knowledge of gene structure and function, and the techniques for DNA manipulation, it is now possible to isolate specific genes and, using vectors based on Agrobacterium Conn., attempt to insert them into the chosen plant species.

A number of techniques have been proposed for DNA transfer into plants, including non-vector induction methods e.g. direct DNA uptake and microinjection of pure DNA. Using immobilised tobacco protoplasts, Crossway et al. (1986) demonstrated that DNA could be taken up and incorporated into the tobacco genome, however only random portions of the DNA were integrated. DNA has also been incorporated into lettuce protoplasts by electroporation (Chupeanu et al. 1989) with more success.

Among plant viruses, the Cauliflower Mosaic Virus (CaMV) has several properties which could prove useful for a gene vector of higher plants. It has a relatively small double stranded genome, is easily manipulated in vitro, cloned viral DNA is infectious when spread onto healthy leaves, and the virus is systemic and is found in high copy number.

Attempts are being made to insert foreign DNA into the CaMV genome and to introduce this DNA into plants through inoculation of susceptible plants. Size

limitations for the insertion of foreign DNA fragments have been restrictive in the use of the CaMV genome as a viral vector, due to the packaging efficiency reducing as the amount of DNA increases above an optimum amount. The largest fragment that has been transferred to plant cells is a 234 base pair fragment containing the dihydrofolate reductase (DHFR) gene (Brisson et al. 1984).

The simplest and most successful vector system to date involves the use of the bacterium Agrobacterium tumefaciens (Smith and Townsend) Conn.

In 1944, Avery, MacLeod and McCarty demonstrated that DNA was the transforming principle in bacteria (Pneumococcus); however, attempts to transform plants with DNA, now known to be the carrier of genetic information, were unsuccessful. In early experiments, germinating seeds and other plant tissues were generally exposed to large amounts of DNA (Ledoux 1975). Most reports of transformation were not reproducible, however, under stringent aseptic conditions (Kleinhofs and Behki 1977). A number of reasons exist for initial failure in transformation attempts, among which is the failure to detect sequences which did actually enter the host genome. Where detection required gene expression, the correct control sequences would have to have been present to allow accurate transcription and translation, and subsequent detection of the gene product. Failure could also have been due to inability of the DNA to

integrate, and therefore it was lost during subsequent multiplication of the host cells.

With the elucidation of events leading to crown gall formation, the potential of A. tumefaciens as a transformation system was recognised. A. tumefaciens is the causal agent of the plant cancer known as crown gall. Infection occurs in response to wounding of the plant in the presence of the pathogen, resulting in a tumour of multiplying undifferentiated cells. The host range of crown gall is usually presented as being very wide, vague and in some cases restricted to the dicotyledonous class of the angiosperms. A comprehensive list of susceptible plant species, though probably not complete, is given by De Cleene and De Ley, (1976).

The first step in realising the potential of Agrobacterium as a gene vector, may have come in 1974 when Zaenen et al. reported that pathogenic strains of A. tumefaciens contain a large plasmid responsible for tumour induction in infected cells. Before this plasmid was detected, White and Braun (1941) proposed that a tumour inducing principle as of then unknown had been responsible for tumour induction in infected cells. Many studies had been carried out with little success, in an attempt to determine exactly what this tumour inducing principle was. Among the factors studied included the examination of various sterile bacterial fractions which were tested for pathogenicity with no reproducible results (Gribnau 1965 and Bieber and Sarfert 1968). The role of growth substances was investigated and although

they increased growth rate of normal plant cells, they failed to induce tumours (White and Braun 1941). Other investigations into the possible role of phage involvement were carried out by a number of workers (Beardsley 1962, Heberlein and Lippincott 1965 and Heberlein and Lippincott 1967a,b.).

Once the presence of the plasmid had been demonstrated, it was investigated as the possible transforming principle of Agrobacterium. It was not possible to demonstrate the presence of the plasmid in avirulent cells of the bacterium and also when the bacterium was cured of the plasmid, virulence was lost (Chilton et al. 1974).

It was demonstrated by Chilton et al. (1977) that the molecular basis of crown gall tumorigenesis was the transfer and stable integration of a portion of the Ti-plasmid, the T-DNA, into the nuclear genome of the plant infected with Agrobacterium. To demonstrate this, the entire plasmid DNA from A. tumefaciens strain A277 was digested with a restriction enzyme (Sma I), giving 19 bands, each of which were used as a probe in hybridization studies of DNA from a tobacco tumour clone. Only one band showed a marked change in reassociation kinetics in the presence of tumour DNA. This study showed that only a small portion of the Ti-plasmid was transferred and integrated into the plant tissue. In no case has total DNA of the Ti-plasmid been found in plant tumour cells. Induction of mutations by means such as transposons (Holsters et al. 1980, De

Greve et al. 1981 and Garfinkel and Nester 1980,) and deletions (Hille et al. 1982) demonstrated that the Ti-plasmid contained two separate regions, both of which were essential for transformation. These regions being the transferred DNA (T-DNA) and a large region (40 Kbp) mapping outside the T-DNA known as the virulence (vir) region.

Transformed plant cells show a number of unusual features. They can grow in tissue culture on media devoid of the plant hormones necessary for in vitro growth of normal plant cells and they synthesise a variety of compounds unique to tumours called the opines (Bomhoff et al. 1976). Opine production is of no benefit to the plant cell and hence the bacterium is manipulating the plant to produce a product which it alone can then use as a carbon and nitrogen source. The genes responsible for hormone independent growth and ability to synthesise and utilise opines must therefore be carried on the Ti-plasmid and more precisely the T-DNA fragment which is actually transferred to and maintained in the host plant.

There are two main types of oncogenic plasmids, classed depending on the opines produced. Both the octopine and nopaline plasmids share several regions varying in the degree of DNA homology (Currier and Nester 1976, Drummond and Chilton 1978). The region transferred and stably maintained in the plant nuclear DNA is highly conserved in both types of plasmids and is known as the common sequence (Chilton et al. 1977,

Leemers et al. 1980, Thomashaw et al. 1980, Willmitzer et al. 1980). The common segment of both types of plasmids encode a variety of polyadenylated transcripts (Willmitzer et al. 1982, Willmitzer et al. 1983, Bevan and Chilton 1982, Engler et al. 1981 and Chilton et al. 1978).

Three genes of the common DNA appear to be directly responsible for tumour formation. These genes are called ipt for an isopentenyltransferase, iaa M for a tryptophan monooxygenase and iaa H for an indoleacetamide hydrolase. These genes determine the enzymes involved in the production of the plant hormones isopentenyl-AMP and indoleacetic acid. The gene functions were elucidated by examining mutants. Normal crown gall tumours are unorganised whereas tumours obtained from mutants containing insertions in gene 4 allow root formation on most plants tested (Garfinkel et al. 1981, Joos et al. 1983 and Ooms et al. 1981). Tumours produced by mutants of genes 1 and 2 grow as green calli and sprout both normal and malformed shoots (Garfinkel et al. 1981, Leemans et al. 1982, Joos et al. 1983 and Ooms et al. 1981). In common with what is known about plant growth regulators, the effects caused by expression of gene 4 can be thought of as cytokinin like, so that inactivation might result in low cytokinin to auxin ratio and hence to root formation. Similarly the combined effects of the expression of genes 1 and 2 can be thought of as auxin like, since mutations result in an apparent increase in cytokinin to auxin ratio

which may lead to shoot production (Ooms et al. 1981 and Amasino and Miller 1982). The proliferation of an undifferentiated crown gall tumour must therefore be induced by the combined activities of the products of genes 1, 2, and 4.

DNA sequence analysis of the T-DNA has shown that this is flanked by a direct repeat sequence of 25 bp (Wang et al. 1984, Yadav et al. 1982 and Zambryski et al. 1982). These sequences are required in cis (Yadav et al. 1982) for T-DNA transfer. By deletion analysis (Fraley et al. 1985) of sequences in the T-DNA, it has been shown that these 25 bp borders are the only DNA sequences in this region required to program T-DNA transfer into plant cells. The border sequences are the recognition sequences for a site specific endonuclease encoded by the virD operon (Yanofski et al. 1986 and Stachel and Nester 1986) and therefore are required for excision. The borders may also be important for the formation of a specific DNA conformation required to allow entry into the plant. The border sequences must therefore flank any DNA to be transferred into the plant cells.

Extensive deletions have been made at each end of the T-region in order to determine if each end was functionally equivalent. A deletion at the right end made the T-region virtually avirulent on most plant species (Holsters et al. 1980, Joos et al. 1983, Ooms et al. 1982b, Wang et al. 1984). By contrast deletions of the left end had no apparent effect on the tumour

forming ability of the T-region (Joos et al. 1983 and Wang et al. 1984).

The second portion of the Ti-plasmid essential for tumour formation is the virulence (vir) loci. The expression of operons in this loci are signalled by plant phenolics such as acetosyringone (Engstrom et al. 1987 and Stachel et al. 1985). The DNA sequence of the vir region has not been found in any established tumour lines and therefore it cannot be essential for tumour maintenance (Leemers et al. 1980, Thomashow et al. 1980, De Beuckeleer et al. 1981 and Ooms et al. 1982a). However, inactivation of genes in the vir region (Klee et al. 1983) demonstrates that it is involved in both the transfer and stable integration of the T-DNA into the plant genome. The Ti-plasmid virulence loci are located in a large 40 kb contiguous region of the Ti-plasmid. There are seven operons named virA, virB, virC, virD, virE, virF and virG, which provide products required for recognition of plant hosts and subsequent T-DNA transfer. Vir expression is tightly regulated. In vegetatively growing bacterial cells only virA and virG are constitutively expressed to any significant level; however, when Agrobacterium is grown in the presence of susceptible plant cells, expression of the other operons become induced to high levels. (Engstrom et al. 1987).

The exact role of each operon is uncertain, but certain functions have been linked to the protein products of particular operons. The virA protein is present in the bacterial inner membrane (Leroux et al.

1987 and Melchers et al. 1989) and is therefore thought to function as a sensor in the recognition of plant signal molecules such as acetosyringone, and subsequently activates virG to a functional level, which in turn activates the other operons. Once the operons have been switched on, an early step in the transfer process must be cleavage of the DNA. In 1985, Koukolikova-Nicola et al. reported the presence of circular intermediates before transfer and integration of the T-DNA into plant DNA. The circular intermediate junction occurs precisely within the 25 bp terminus sequence essential for DNA transfer. The virD operon has been found to encode two proteins called VirD1 and VirD2 (Stachel and Nester 1986) which together determine an endonuclease which cleaves the T-DNA at site specific areas within the border sequences (Albright et al. 1987).

The exact form in which the cleaved DNA is transferred is uncertain since both T strands (Stachel et al. 1986) and double stranded T molecules (Jayaswal et al. 1987) have been found. VirD2 and VirE2 proteins have been found to bind to T-strands and, therefore, it is not thought that transfer of DNA occurs alone (Herrera-Estrella et al. 1988, Ward and Barnes 1988a and Gietl et al. 1987). The virB operon codes for proteins which are exported to the membrane (Thompson et al. 1988 and Ward et al. 1988b) and appear to be involved in the formation of a structure through which the actual transfer occurs. The remaining vir loci virC, virE and

virF, known as host-range loci, are required for the formation of tumours on only certain plant species (Otten et al. 1985 and Melchers et al. 1990).

In addition to regions on the Ti-plasmid, it has been shown by Tn5 insertion into chromosomal DNA that there are chromosomal genes which are required for virulence (Garfinkel and Nester 1980). Chromosomal genes are probably involved in the attachment of bacteria to plant cells, an early step in tumor formation (Lippincott and Lippincott 1969).

Interactions between plant cells and Agrobacterium initiates a chain of events leading to transfer of the T-DNA from the Ti-plasmid into the plant nuclear DNA.

As Agrobacterium tumefaciens appears to be a natural "genetic engineer" the major objective in the use of Agrobacterium is to take advantage of these natural properties in order to introduce and express new genes in plants. Agrobacterium rhizogenes, a closely related pathogen containing a root-inducing plasmid (White et al. 1982), infection by which leads to hairy root disease, has also been exploited in plant transformation (Morgan et al. 1987, Guerche et al. 1987, Brillanceau et al. 1989 and Guellec et al. 1990).

The first step in the expression of foreign genes in plants is the construction of genes with the correct control signals known to function in plant cells. The correct transcriptional and translational control sequences must be available for information transfer from the gene into the corresponding phenotype. One of

the best candidates for use as a donor of transcriptional signals is the nopaline synthase (nos) gene. Since the opine genes are known to be expressed normally in transformed callus tissue and plant tissues regenerated from calli containing an opine gene (Otten et al. 1981 and De Greve et al. 1982a), control sequences recognised by the host must be present.

Analysis of the nucleotide sequences of the nos promoter (Depicker et al. 1982, Bevan et al. 1983a), reveals sequences homologous to the TATA box, an AT rich region 25-35 bp upstream from the initiation site and a sequence similar to the AGGA box consensus sequence for plant genes 60 to 80 bp upstream from the 5 prime end of transcription (Dobson et al. 1982).

There are two main types of vector system in use based on the properties of the Ti-plasmids. The first one is designed to cointegrate into resident Ti-plasmids, and the other system is a binary system where the genes of interest are on a separate plasmid which replicates autonomously.

However, before I discuss the two systems I will mention a disadvantage associated with the use of the Ti-plasmid. This disadvantage of DNA transfer using Agrobacterium is that the high level of phytohormones produced in transformed tissue, due to expression of genes on the T-DNA, prevents cell regeneration into whole plants. To overcome this difficulty, disarmed Ti-plasmids (Zambryski et al. 1983) with partial or total deletion of the phytohormone encoding genes have

been constructed and can be used to transfer DNA without inhibiting regeneration of transformed cells. Because both the presence of opines and the ability of transformed cells to proliferate without added hormones were traits used to indicate successful infection-transformation, the use of disarmed Ti-plasmids necessitated the development of selectable markers. The vectors also require a selectable marker useful for following the introduction of the vector into Agrobacterium.

The two vector systems in use were developed to overcome difficulties encountered in the use of the Ti-plasmids as cloning vehicles. Foreign genes could not be inserted directly into the Ti-plasmids due to their large size (approx. 140-230 Kb) making isolation and manipulation difficult. Construction of plasmids identical to the Ti-plasmid was also difficult due to the large number of functions required for T-DNA transfer. As mentioned earlier, however, the T-DNA is delimited by direct 25 bp repeats which are the only sequences required in cis to cause T-DNA excision. A transformation vector must, therefore, only contain one or both borders or be capable of cointegrating in such a manner that it becomes flanked by borders.

In 1981 Leemans et al. developed a vector able to replicate in both Agrobacterium and E. coli. Such plasmids were called cointegrate or intermediate vectors. Cointegrating vectors (intermediate vectors), capable of replication in both Agrobacterium and E. coli

carry a subfragment of the T-DNA to allow homologous replication between the vector plasmid and the Ti-plasmid. Briefly, these vectors contained a fragment of DNA carrying the kanamycin resistance gene, and an origin of replication from the broad host range W-plasmid Sa (Wantabe et al. 1968) which replicates in both E. coli and A. tumefaciens, and a defined fragment from the T-region of a Ti plasmid with specific restriction enzyme cleavage sites. By in vitro recombination techniques, DNA sequences were inserted into the cloned T-region fragment. The intermediate vector (iv) was subsequently introduced into Agrobacterium and the manipulated T-region fragment was exchanged for the homologous fragment of a resident Ti-plasmid by in vitro recombination. In plants inoculated with Agrobacterium harbouring the vector, the total T-region was found integrated in plant DNA. Here a smaller vector was constructed which could be manipulated in E. coli and transferred into Agrobacterium where it underwent homologous recombination and allowed the required sequence to become part of the Ti-plasmid. The requirement for homology to allow recombination means that the vector was capable of integrating into a limited number of Ti-plasmids. A plasmid which contained the chosen selectable marker gene along with a bacterial antibiotic resistance gene selectable in Agrobacterium was introduced into these cells by transformation (Matzke and Chilton 1981) or conjugation (Van Haute

et al. 1983). Subsequent replacement of the normal T-DNA segment with the altered segment containing the inserted DNA occurred by homologous recombination inside the Agrobacterium. Because of the low frequency of the recombination event between the intermediate vector and the Ti-plasmid, construction of plasmids was quite complex.

The first expression of inserted genes occurred in 1983 when Herrera-Estrella et al. (1983b) constructed a chimaeric gene linking the nos promoter sequence to the coding sequence of the octopine synthase enzyme. This was inserted into an iv and recombined with an acceptor plasmid, and expression of the gene subsequently occurred. The construct consisted of both the nos promoter and polyadenylation sites and the coding sequence of the chloramphenicol acetyltransferase (cat) enzyme.

Similar constructs using the bacterial neomycin phosphotransferase II gene were reported. This gene confers resistance to antibiotics kanamycin, neomycin and G-418 by inactivating them by phosphorylation (Herrera-Estrella et al. 1983b).

Two cointegrating systems are in use today. The first utilises the disarmed Agrobacterium plasmid pGV3850 (Zambryski et al. 1983). In this plasmid the phytohormone genes of the C58 plasmid have been excised and replaced by the pBR322 sequence. Any plasmid containing the pBR322 sequence homology can be cointegrated into the disarmed Ti-plasmid. The second

cointegration system involves removal of all the phytohormone genes from the Ti-plasmid. A left border and a small part of the original T-DNA referred to as the limited internal homology remain intact. The vector to be introduced into Agrobacterium contains the LIH region for homologous recombination as well as a right border. The cointegration constructs a functional T-DNA with the left and right borders (Fraley et al. 1985).

The aim of the cointegrate vector system has been to generate an intact Ti-plasmid. However, this is not necessary for infection and transformation. Hoekema et al. 1983 reported the interaction of two compatible plasmids, one containing the vir-region and the other carrying the T-DNA on a wide host-range replicon. As the two plasmids showed no homology, no cointegration could have occurred by homologous recombination.

The vir region of the Ti-plasmid can provide all the mobilization functions to the DNA in trans, that is on a second plasmid (Hoekema et al. 1983). This led to the development of binary vectors (Bevan 1984). One plasmid carries the foreign gene to be introduced into plant cells between the two T-DNA borders, and the other carries the vir functions required for transfer of the DNA. Many vectors contain two borders, in which case, only the DNA sequence between them will be transferred. However, a single border is sufficient for transfer (Horsch and Klee 1986). In this case, transfer initiates at the single border and the entire plasmid becomes the T-DNA. A major advantage of the binary vector system is

its lack of dependence on a specific Ti-plasmid. This system can again be modified by deleting the genes for growth regulator autonomy and so a marker gene is required to detect transformation (Simpson et al. 1986). The binary vector system is the vector which I have used in the development of a transformation system for soft fruit species and will be discussed further in chapter 5.

As mentioned above, selectable and scorable marker genes are necessary today in most plant transformation experiments due to deletion of characteristics which previously indicated the insertion of the T-DNA. The use of selectable markers will be discussed in detail again in chapter 5.

The ultimate aim of the introduction of new genetic material into the plant is to obtain a transformed whole plant in which the genetic material is stably maintained. As described in chapter 2 (concerned with the production of galls on Rubus, Ribes and Vaccinium spp., to ensure Agrobacterium is suitable as a vector in soft fruit), whole plant inoculation is not useful as only the cells at the site of infection will contain the foreign DNA. The infection technique is therefore very important and must be considered in conjunction with the regeneration methods available (if any) for that particular plant species. The infection techniques used to date will be discussed in chapter 2. For soft fruit species no suitable regeneration techniques were available, the development of which constituted a

fundamental step in the transformation process. The development of regeneration techniques for Rubus, Ribes, Vaccinium and Fragaria spp. will be described in chapters 3 and 4.

A transformation system for soft fruit could potentially have immense implications on the improvement of soft fruit spp. Specific characteristics, (for which the gene sequence would have to be isolated and cloned) could be introduced into important cultivars, substantially quicker than by plant breeding techniques and without the co-transfer of undesirable characteristics associated with breeding. Genes which do not naturally occur within the soft fruit could also be inserted, as well as gene transfer occurring between the soft fruit spp.

Presently the use of Agrobacterium as a vector has yielded some positive result in that first, it has been shown that Mendelian transmission of the genes introduced into the Ti-plasmids occurred (Otten et al. 1981), and secondly, morphologically and functionally normal plants have been obtained from plant cells transformed with Agrobacterium tumefaciens strains harbouring a Ti-plasmid (De Greve et al. 1982a).

The following chapters are concerned with the development of an appropriate transformation system in soft fruit species.

Chapter 2.

The inoculation of plant material with seven isolates of Agrobacterium species, to determine its usefulness in the development of a transformation system for soft fruit.

The inoculation of plant material with seven isolates of Agrobacterium species, to determine its usefulness in the development of a transformation system for soft fruit.

Introduction

Bacteria from the genus Agrobacterium are gram-negative motile rods found in soils throughout the world. They are not obligate pathogens and can remain viable in the soil for a long period of time. They have been subdivided into three classes: those inducing crown galls are called Agrobacterium tumefaciens; those inducing "hairy root" disease, A. rhizogenes; and the non-pathogenic strains of A. radiobacter. Classification on the basis of pathogenic characteristics is rather difficult, however, as the ability to infect and produce symptoms is encoded by DNA carried mainly by plasmids which are mobile and move between classes.

The host range of crown gall appears to be very wide. De Cleene and De Ley (1976) have reviewed the susceptibility of 1193 plant species belonging to 588 genera and 138 families. Plant infection, leading to tumour formation, occurs when the bacterial cells come into contact with a wound site on the plant, inducing attachment of the bacteria to the exposed cells (Lippincott and Lippincott 1969). The plant cell does not appear to play an active part in the process of attachment (Matthysse et al. 1978), however (as

described in Chapter 1) the wounded plant gives out signals (e.g. phenolic compounds) to the bacterium, inducing it to bind and infect. It is not known if binding occurs only to certain types of plant cells exposed in the wound or to all the exposed cells. Attachment of the bacterium to the plant cell probably occurs by some protein product of the chromosomal virulence genes (Douglas et al. 1985). The bacterial cells then multiply within the host tissues, some bacteria transferring a portion of plasmid DNA, the T-DNA, into host cells. This T-DNA becomes integrated into the plant chromosome and replicates with the host DNA (Chilton et al. 1977, Gurley et al. 1979 and Wullems et al. 1981). Plant nuclear DNA of transformed plant tissues contains approximately 20 Kb of the Agrobacterium Ti-plasmid covalently integrated into its chromosome.

The exact mechanism of transfer is unknown but it has been postulated that it must involve either the use of some structure which protrudes from the bacterial surface e.g. pili, or a fusion of the bacterium with the plant cell plasmalemma (Matthysse 1986). From the evidence produced by analysis of the function of the virB operon, it would appear that DNA transfer is through some membrane structure (Thompson et al. 1988). As described in Chapter 1, the transfer of DNA is under the control of the vir gene region of the T-DNA, which is activated by some signal from the wounded plant. Integration of the T-DNA then occurs, the two

border fragments (consisting of twenty five base pair direct repeats) playing an important role in both transfer and integration. It has been shown that only the right border is essential for DNA transfer. The expression of the T-DNA in the host cell results in an alteration of hormone levels in plant cells, resulting in the formation of tumours (Amasino and Miller 1982).

The morphology of a tumour depends on the levels of auxin and cytokinin induced by the T-DNA in the plant cells.

Tumour formation can be used to determine if Agrobacterium tumefaciens infection has occurred (or hairy root production in the case of A. rhizogenes), provided a non-disarmed or wild type strain of bacterium is used. This wild type strain will contain the genes responsible for tumour formation, which when transferred into, and expressed in plant cells, cause a severe disturbance of the natural phytohormone balance at the site of infection and results in proliferation of both transformed and non transformed cells.

Before Agrobacterium could be used in the development of a vector system for soft fruit, it had to be demonstrated that Agrobacterium would actually infect plant material in tissue culture. It was known that crown gall disease occurs on Rubus spp. in the field (McKeen 1954). To a lesser extent crown gall also occurs on Ribes spp. in the field. This was first reported in Hungary in 1976 (Sule and Sass 1977). However I could not locate any field report of crown gall on Fragaria or

Vaccinium spp. The tissue culture environment, however, is different from the natural environment and crown gall infection may be affected. With inoculation occurring in a tissue culture environment, McNicol and Schwartz (pers. commn. 1987) found only 0.025% infection of Rubus spp. with Agrobacterium. With Fragaria spp. McNicol and Hyman (pers. commn. 1987) found that Agrobacterium would infect in tissue culture. No report of crown gall on Ribes or Vaccinium in tissue culture exists.

Therefore the ability of Agrobacterium to cause galling (as an indication of infection) on Rubus, Ribes and Vaccinium had to be determined as an initial step in the development of a transformation system for soft fruit using Agrobacterium.

The assessment of infectivity of various Agrobacterium isolates on Rubus spp.

To assess the infectivity of Agrobacterium on Rubus spp., four A. tumefaciens isolates and three A. rhizogenes isolates were used. The formation of either galls (A. tumefaciens) or hairy roots (A. rhizogenes) was used to determine if the plants were susceptible to the particular isolate used. Before a large scale inoculation of plant material with all seven isolates of Agrobacterium was attempted, three preliminary investigations were carried out to determine first the optimum inoculation procedure, second the optimum incubation time of the explant and Agrobacterium, and

third if any specific conditions of pH were required. For crown gall formation, a number of techniques have been used for inoculation of plant tissues. Hemstad and Reisch (1985) found that piercing shoots with a needle produced more profuse galls than dipping shoot bases or decapitated shoot tips into a bacterial suspension. Other workers have found that injecting a bacterial suspension into a wound site very successfully yields galls (De Cleene and De Ley 1976).

Materials and Methods.

Bacterial Isolates

A. tumefaciens

516
A6
Ach5
T37

A. rhizogenes

Ar2628
Ar2629
Ar9402

The above isolates were kindly supplied by Dr. J. Manners of the John Innes Institute.

Rubus genotypes

Tayberry - Red raspberry x blackberry hybrid
Sunberry - Red raspberry x blackberry hybrid
Loch Ness - Blackberry
8242E6 - Red raspberry

Media Recipes

Luria-Bertani Broth/Medium*

Difco bacto-tryptone	10g
Yeast extract	5g
NaCl	5g
D-glucose	1g
Agar*	10g
pH 7.0	

Ref: Maniatis et al. 1982

Yeast Mannitol Broth/Medium*.

Yeast extract	0.4g
Mannitol	10.0g
NaCl	0.1g
K ₂ HPO ₄	0.5g
MgSO ₄ .7H ₂ O	2.0g
Agar*	15.0g
pH 7.0	

Ref: Hooykaas et al 1977

*: Solid medium.

Liquid M+S based Plant Medium.

	mg/l
NH ₄ NO ₃	1650.0
KNO ₃	1900.0
CaCl ₂ .2H ₂ O	440.0
MgSO ₄ .7H ₂ O	370.0
KH ₂ PO ₄	170.0
FeNa.EDTA	36.70
H ₃ Bo ₃	6.20
MnSO ₄ .4H ₂ O	22.30
ZnSO ₄ .7H ₂ O	8.6
KI	0.83
Na ₂ MoO ₄ .2H ₂ O	0.25
CuSO ₄ .5H ₂ O	0.025
CoCl ₂ .6H ₂ O	0.025
glutamine	200.0
inositol	200.0
sucrose	30000.0
pH 5.6	

Solid Plant Medium-Raspberry multiplication medium(RM).

	mg/l
NH ₄ NO ₃	1650.0
KNO ₃	1900.0
CaCl ₂ .2H ₂ O	440.0
MgSO ₄ .7H ₂ O	370.0
KH ₂ PO ₄	170.0
FeNa.EDTA	36.70
H ₃ Bo ₃	6.20
MnSO ₄ .4H ₂ O	22.30
ZnSO ₄ .7H ₂ O	8.6
KI	0.83
Na ₂ MoO ₄ .2H ₂ O	0.25
CuSO ₄ .5H ₂ O	0.025
CoCl ₂ .6H ₂ O	0.025
Agar	8000.0
Biotin	0.1
Calcium pantothenate	1.0
Charcoal (activated)	20000.0
Cysteine-HCl	1.0
Glutamine	200.0
Glycine	4.0
Inositol	200.0
Nicotinic acid	1.50
Pyridoxin-HCl	1.50
Sucrose	30000.0
Thiamine-HCl	1.1
pH 5.6	

Ref: This medium was adapted from Carrie et al. 1979.

General procedures for inoculation.

Preparation of inoculum.

The inoculum was prepared by introducing a loopful of the bacterial isolate into 10 ml of the appropriate broth (LB for A. tumefaciens and YM for A. rhizogenes). This was incubated overnight at 28°C in a shaking water bath. After 24 hours, the culture was pelleted by centrifuging at 10,000 rev min⁻¹ for five minutes and resuspended in liquid M+S based plant medium with 30 g/l sucrose. This was again pelleted and resuspended in liquid plant media.

Preparation of plant material.

Stem segments of approximately 2 cm in length and containing approximately 3 axillary shoots were obtained for inoculation.

All explant material for the experiments was obtained from in vitro grown plantlets, maintained at 20°C under warm white fluorescent tubes at 70 $\mu\text{mol m}^{-2}\text{s}^{-1}$ for 16 hour light:8 hour dark on solid raspberry multiplication medium. All stock cultures were routinely subcultured at 5 week intervals. For a given experiment all explants were derived from plantlets subcultured on the same date.

The stem segments were prepared for inoculation by wounding the stem once with a sterile scalpel blade under a dissecting microscope. The stem segments were chosen where possible to contain the same number of shoots on each segment, generally (as mentioned above) 3 shoots per segment.

Incubation conditions

After inoculation, the plant material and bacteria were incubated on sterile filter paper discs which had been dipped into liquid plant media and placed in a petri dish.

Incubation time of the explant and Agrobacterium was from 1-6 days as determined by the preliminary experiment on plant survival.

Inoculation of plant material.

The Agrobacterium isolate resuspended in the plant basal medium was poured into a 9 cm diameter petri dish. The wounded plant material was placed into the suspension for 20 mins, before being removed and placed onto sterile filter paper for incubation. The sterile filter paper was dipped into raspberry multiplication media without hormones, agar or charcoal. After the incubation period of the explant and the bacteria was complete, the plant material was dipped into 400 mg/l of the antibiotic carbenicillin before being placed onto

raspberry multiplication media, and placed under fluorescent tubes at $70 \text{ umol m}^{-2} \text{ s}^{-1}$ at 20°C .

Gall production was assessed after a period of 20 weeks.

Control plants.

A number of explants were used as controls. These were subjected to exactly the same conditions as the inoculated plants, being wounded in exactly the same way, dipped into plant liquid media without Agrobacterium and incubated under exactly the same conditions. In no case was Agrobacterium involved in preparation of the controls.

Preliminary investigations.

(N.B. The genotype Tayberry was used in all preliminary experiments).

1. Determination of the most effective procedure for inoculation.

Procedures

Tayberry shoots of approximately 2 cm in length were obtained and treated as follows:

1. Shoots were wounded once with a sterile scalpel blade and placed into a bacterial suspension held in a petri dish for 20 mins.
2. A needle was dipped into the bacterial suspension and used to pierce the plant material in one place.
3. As number 2, only explants were placed directly onto plant solid media without dipping into the antibiotic solution.
4. A fine needle was dipped onto a petri dish of the bacterium and used to pierce the plant once.

Fifteen plants were treated at each of the inoculation procedures using the Agrobacterium isolate 516. This isolate was chosen initially as it was isolated from raspberry plants in the field at SCRI.

The plants were incubated with Agrobacterium for 48 hours for procedures 1, 2 and 4 before being dipped into a solution of the antibiotic carbenicillin (400 mg/l) for 10 mins, blotted on sterile filter paper and placed onto plant solid media. If Agrobacterium contamination became noticeable after the plants had been placed onto solid media, the plants were removed and dipped into a solution of the antibiotic and placed onto fresh media. Explants which had been placed directly onto plant media without dipping in a solution of carbenicillin became

heavily contaminated with Agrobacterium within 1 week, and required frequent dipping into carbenicillin to remove the infection and fresh media after each dip.

Results Preliminary Exp.1.

Table Pl. Effect of 4 inoculation procedures on gall production.

Procedure	No. plants	No. galls	% gall prodn.
1	15	6	40
2	15	5	33
3	15	7	47
4	15	5	33

This first experiment demonstrated the ability of Agrobacterium to induce galls on tissue culture material.

No significant differences occurred between the inoculation procedures, all proving similarly effective.

2. Determination of the effect of various incubation periods with Agrobacterium on plant survival.

The survival of plant material with Agrobacterium at various incubation times was examined to find out how long the plant material would survive and remain viable. Two different isolates were used here. Inoculation occurred as in procedure 1 of the preliminary investigation.

Results Preliminary Exp.2.

Table P2. Effect of incubation time on inoculated explant survival.

Plants	Incubation Time(days)	Number of plants alive		% of plants alive	
		Ach 5	A6	Ach 5	A6
30	1	30	30	100	100
30	2	29	30	97	100
30	4	30	22	100	73
30	6	27	28	90	93
30	8	14	12	47	40
30	10	10	14	33	47

Plant survival was examined after 4 weeks, with no further plant deaths occurring after this time. Generally the plants died within 1 week of being placed onto growth medium. No significant differences occurred between 1, 2, 4 and 6 day incubation periods; however, explant survival was greatly reduced when incubated for 8 or more days.

3. Determination of most efficient pH for plant infection.

To determine if Agrobacterium required a specific pH at which infection of the plant material would occur to a greater extent, inoculation and incubation was conducted in plant liquid medium, with explants placed onto solid raspberry medium at pH 5.3, 5.6, 6.0 and 6.5. Again inoculation occurred as in procedure 1 of the preliminary investigation for determination of the most

effective inoculation procedure. Incubation of the explant with Agrobacterium was for a period of 2 days.

Results Preliminary Exp.3.

Table P3. Effect of 4 different pH values on gall production.

516	No.of plants inoc.	pH	No. with galls	%
	25	5.3	13	52
	24	5.6	10	42
	26	6.0	11	42
	25	6.5	9	36

As can be seen from the results above, very little difference in gall production occurred at different pH values, although gall production was greater at the lowest pH.

From the results of three preliminary investigations conducted, decisions were made on the conditions for inoculation.

Because no significant differences existed between the inoculation treatments, treatment 1 was chosen as it could be carried out more conveniently and quickly.

When the effect of time on the incubation of the explant and bacterium was examined, plant material survived and grew well with incubation times of up to and including 6 days. Significant differences occurred between a time of 8 or 10 days and the other times used, but between times of 1, 2, 4 and 6 days, there were no significant differences in plant survival.

No significant differences in gall production were observed due to pH, and the pH value of 5.6 was therefore chosen, as this is generally used for plant tissue culture of Rubus spp. The variation which occurred in the 3 preliminary experiments was found not to be significant using a Chi-squared goodness of fit test.

Experiment 2.1.

Assessment of 7 Agrobacterium isolates for their ability to produce crown galls or hairy roots.

The assessment of virulence of the 7 isolates was carried out using inoculation procedure 1, at a pH of 5.6 and with incubation times varying from 1-6 days. All other conditions e.g. temperature (20°C), age of plant material (used at week 3 of culture cycle), flow cabinet and lighting conditions ($70 \text{ umol m}^{-2} \text{ s}^{-1}$), were maintained constantly throughout the experiments. The genotype Tayberry was again used in the assessment.

Results Experiment 2.1.

Table 2.1. Gall (or hairy root) producing ability of 7 different Agrobacterium isolates.

Isolate	No.of plants inoc.	Co-inc. time days	No.with galls or hairy roots.	%
516	15	1	5	33
	15	2	6	40
	14	3	7	50
	16	6	4	25
Ar 2629	13	1	12	92
	12	2	11	92
	12	3	12	100
	13	6	13	100
Ar 2628	13	1	10	77
	13	2	11	85
	12	3	10	83
	12	6	9	75
A 6	10	1	7	70
	12	2	9	75
	13	3	9	70
	14	6	8	57
ACH 5	15	1	15	100
	13	2	10	77
	14	3	8	57
	15	6	8	53
Ar 9402	15	1	11	73
	14	2	12	86
	15	3	15	100
	13	6	13	100
T 37	15	1	No results were obtained as the plant material all died.	
	15	2		
	13	3		
	14	6		

Using Genstat, a binomial model was fitted to the "number of galls" and the isolate effect was found to be highly significant ($P < 0.001$).

Experiment 2.2.

Effect of isolates Ar9402 and Ach5 on a wider range of genotypes.

Ach5 and Ar9402 which were particularly successful in causing gall or hairy root production on Tayberry, were used to infect a further 3 Rubus genotypes of diverse genetic makeup to determine if similar results could be obtained.

Results Experiment 2.2.

Table 2.2a. Ability of Ach5 to induce galls on 3 Rubus genotypes.

Genotype	Ach5 1 day coincubation		
	No. plants	No. galls	% galls
Sunberry	20	19	95
8242E6	20	17	85
Loch Ness	20	19	95

Table 2.2b. Ability of Ar9402 to induce hairy roots on 3 Rubus genotypes.

Genotype	Ar9402 3 days coincubation		
	No. plants	No. hairy roots	% hairy roots
Sunberry	20	19	95
8242E6	20	17	85
Loch Ness	20	19	95

No significant differences in gall forming ability occurred due to the plant genotypes used. The effect of isolate Ach 5 following one day of co-incubation was examined using chi-squared: the results showing that a specific isolate would be expected to produce the same amount of galls on the 3 genotypes.

Experiment 2.3.

The inoculation of Ribes nigrum with Ach5 and Ar9402.

Materials and methods

Blackcurrant solid medium (BM)

	mg/l
NH ₄ NO ₃	1650.0
KNO ₃	1900.0
CaCl ₂ .2H ₂ O	440.0
MgSO ₄ .7H ₂ O	370.0
KH ₂ PO ₄	170.0
FeNa.EDTA	36.70
H ₃ BO ₃	6.20
MnSO ₄ .4H ₂ O	22.30
ZnSO ₄ .7H ₂ O	8.60
KI	0.83
Na ₂ MoO ₄ .2H ₂ O	0.25
CuSO ₄ .5H ₂ O	0.025
CoCl ₂ .6H ₂ O	0.025
Agar	6.0
Benzylaminopurine	1.0
Glycine	2.0
Inositol	200.0
Nicotinic acid	1.0
Pyridoxine-HCl	1.0
Sucrose	20000.0
Thiamine-HCl	0.5
pH 5.6	

Liquid medium

	mg/l
NH ₄ NO ₃	1650.0
KNO ₃	1900.0
CaCl ₂ .2H ₂ O	440.0
MgSO ₄ .7H ₂ O	370.0
KH ₂ PO ₄	170.0
FeNa.EDTA	36.70
H ₃ BO ₃	6.20
MnSO ₄ .4H ₂ O	22.30
ZnSO ₄ .7H ₂ O	8.60
KI	0.83
Na ₂ MoO ₄ .2H ₂ O	0.25

CuSO ₄ .5H ₂ O	0.025
CoCl ₂ .6H ₂ O	0.025
Glycine	2.0
Inositol	100.0
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Sucrose	20000.0
Thiamine-HCl	0.1
pH 5.6	

Blackcurrant medium based on M+S salts with additions.

Procedure

The infection procedure was carried out as for Rubus spp. Explants of 2 cm in length and containing 3-5 axillary buds were obtained and inoculated with Ach5 and Ar9402. Again a single wound was made in the stem, and the explant was placed in a petri dish containing an overnight culture of Agrobacterium resuspended in blackcurrant liquid medium at pH 5.6. Co-incubation time was 1 day for Ach5 and 3 days for Ar9402.

Results Experiment 2.3.

Table 2.3a. Ability of isolates Ach5 and Ar9402 to induce galls (Ach5) or hairy roots (Ar9402) on the blackcurrant cultivar Ben More.

	No. plants	Ben More No. with Galls or hairy roots	% Galls or hairy roots
Ach5	30	25	83
Ar9402	30	27	90

Table 2.3b. Ability of isolates Ach5 and Ar9402 to induce galls (Ach5) or hairy roots (Ar9402) on the blackcurrant cultivar Ben Sarek.

	No. plants	Ben Sarek No. with Galls or hairy roots	% Galls or hairy roots
Ach5	30	27	90
Ar9402	30	28	93

The above results demonstrate that Agrobacterium infects blackcurrants giving a high level of galling (or hairy roots).

Experiment 2.4.

The inoculation of *Vaccinium corymbosum* with Ach5.

Materials and methods

Woody plant medium (WPM)

	mg/l
NH ₄ NO ₃	400.0
Ca(NO ₃) ₂ ·4H ₂ O	556.0
K ₂ SO ₄	990.0
MgSO ₄ ·7H ₂ O	370.0
KH ₂ PO ₄	170.0
CaCl ₂ ·H ₂ O	96.0
MnSO ₄ ·H ₂ O	16.9
ZnSO ₄ ·7H ₂ O	8.6
CuSO ₄ ·5H ₂ O	0.25
H ₃ BO ₃	6.2
NaMoO ₄ ·2H ₂ O	0.25
FeSO ₄ ·7H ₂ O	27.8
Na ₂ ·EDTA	37.3
Agar	6.5
Dimethylallylamino-purine	3.0
Glycine	2.0
Inositol	100.0
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Sucrose	20000.0
Thiamine-HCl	1.0
pH 5.3	

Ref:Lloyd and McCown 1980.

Liquid WPM as above without agar or Dimethylallylamino-purine, pH 5.3.

Procedure

Blueberry inoculation was effected in a similar way to Rubus inoculation. Sterile plant material of approximately 2 cm in height was wounded twice and placed into a petri dish containing Agrobacterium resuspended in liquid WPM. Two very shallow wounds were made in the blueberry stem due to the stems being substantially thinner than those of both Rubus and Ribes spp. Only Ach5 was used for inoculation, due to the small numbers of blueberries in culture at that time and co-incubation was for 1 day.

Results Experiment 2.4.

Table 2.4. Ability of Agrobacterium isolate Ach5 to induce galls on 2 blueberry cultivars.

Cultivar	Ach5	
	Galls	% Galls
North Sky	23/30	77
North Country	24/30	80

Similar results were obtained for blueberries as for the other species examined, giving high levels of galling.

The following figures show crown gall disease on tissue culture plantlets of Vaccinium cv. North Country (2.1) and Rubus cv. Tayberry (2.2) and hairy root disease on Ribes cv. Ben More (2.3).

Fig. 2.1

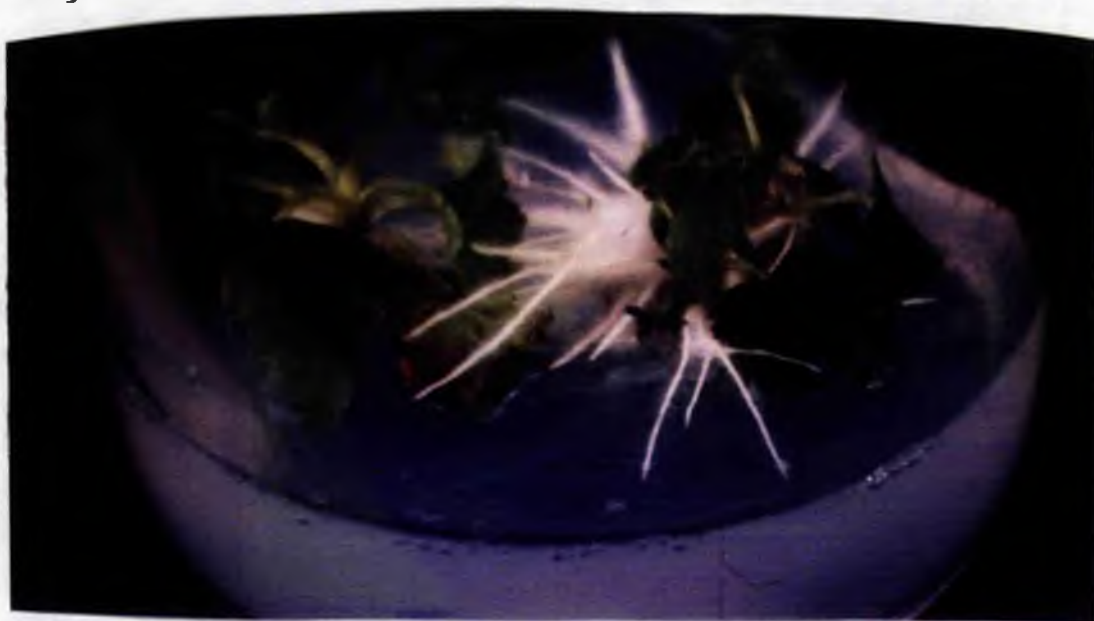


Fig. 2.2



Crown Gall disease on the blueberry cv. North Country (2.1) and the hybrid berry cv. Tayberry (2.2).

Fig. 2.3



Hairy Root disease on the blackcurrant cv. Ben More (2.3).

Discussion

The optimum inoculation conditions for infection were determined by three preliminary investigations. The first involved determination of the optimum inoculation procedure. Four relatively easy methods of inoculation were compared for ability (if any) to result in the formation of galls or hairy roots. Procedure 1 involved making a wound with a scalpel blade and putting the explant into a suspension of the bacterium. The other three procedures involved the use of a fine needle which had been dipped into either a suspension of Agrobacterium or into a plate of Agrobacterium and then used to wound the plant in one place. In three of the procedures the plant material was incubated with the bacterium for 24 hours before being placed into a solution of the antibiotic carbenicillin and then placed onto solid raspberry multiplication medium. In the remaining treatment, the plant was placed directly onto solid raspberry medium after wounding and inoculation had occurred. An incubation period of 24 hours was chosen on the assumption that this would be sufficient time for Agrobacterium infection to occur but insufficient for the incubation conditions to damage the plant.

The results from this investigation showed that gall production did occur in tissue culture on Rubus spp. with very little variation observed in the percentage of

galls produced over treatments. As procedure 1 could be carried out most easily and quickly on a large number of explants it was chosen as the procedure for inoculation in all further experiments.

The second preliminary experiment involved determination of the optimal incubation conditions of the explant and Agrobacterium. Conditions were required whereby Agrobacterium would have time to infect plant material but which were not too severe to cause damage to subsequent plant growth. I decided to incubate the explant and Agrobacterium on sterile filter paper discs which had been dipped in liquid plant medium. A large number of explants could be incubated in this way, without requiring the frequent change of medium, which is necessary when plant material is put into culture jars containing solid medium, on which the Agrobacterium grows profusely. Few changes in the plants environment were desirable to minimise additional damage to the plant other than that already caused by deliberate wounding. The explants could thus be removed quickly and easily and placed directly into the antibiotic solution.

The results of the incubation experiments showed very little difference in amount of gall production over incubation periods that varied from 1 to 6 days in length. However, increasing the incubation period to 8 days or more significantly reduced % plant survival to 47% with the isolate Ach5 and 40% with A6.

The third preliminary experiment, examined the effect of pH on the ability of the Agrobacterium isolate to

infect an explant. Four pH values were chosen under which the inoculation experiment was carried out. The four pH values were chosen to be between what the plant material is usually grown at (5.6) and the pH for Agrobacterium culture (7.5). Very little effect of pH was detected and consequently a pH of 5.6 was used in all further experiments.

Once the experimental conditions had been defined, a range of 7 Agrobacterium isolates were tested to determine the most virulent. On the basis of crown gall or hairy root production, a significant difference was observed in the ability of the isolates to infect. The results obtained showed that 5 of the 7 isolates infected efficiently giving between 70% and 100% gall production. Of the other 2 isolates, one infected at a level of approximately 37% over all while isolate T37 caused the death of all plant material infected. Control plants, however, which had been subjected to exactly the same conditions, except they were placed into liquid plant media without Agrobacterium survived and grew normally.

These results show very clearly that Agrobacterium infects Rubus spp. in a tissue culture environment. Most isolates were aggressively pathogenic towards Tayberry. Of the 3 A. rhizogenes isolates, only Ar9402 produced hairy roots, both Ar2628 and Ar2629 produced galls.

To confirm that the ability of Agrobacterium to infect in tissue culture was not specific to the hybrid berry Tayberry, three other Rubus cultivars were

infected. These included the hybrid berry cv. Sunberry, the red raspberry selection 8242E6 and the blackberry cv. Loch Ness. The Agrobacterium isolates Ach5 and Ar 9402 were chosen as both could induce 100% gall (or hairy root) production on Tayberry and in addition Ar 9402 was the only A. rhizogenes isolate to produce hairy roots. The results demonstrated that the pathogenicity of the isolates was not specific to certain cultivars in Rubus.

Once it was established that Agrobacterium infected a range of Rubus spp., other soft fruit species were examined for susceptibility to Agrobacterium.

Two blackcurrant cultivars were inoculated with Ach5 and Ar9402 using the same conditions determined for Rubus spp. from the preliminary experiments. In addition two blueberry cultivars, North Sky and North Country were inoculated with Ach5. Crown gall occurred on approximately 77% of all plants inoculated.

These results provide clear evidence that Agrobacterium transfers genes into the plant material tested and that the genes are subsequently expressed, causing a proliferation of plant cells. Gall production is not due to wounding as none of the control plants despite being wounded produced galls. The results show that it is feasible to use Agrobacterium in the development of a transformation system for soft fruit.

Crown gall formation occurred on the inoculated plants only, at the site of wounding. When the area containing the gall(s) was removed, normal plants

regenerated which did not contain galls. This shows that although Agrobacterium does infect Rubus, Ribes and Vaccinium spp., and can therefore be used as a gene vector, the system of inoculation is not of use for gene transfer into a whole plant, as only the cells at the site of infection contain the foreign DNA. Instead an inoculation system is required whereby one or a few cells of plant material can be inoculated with Agrobacterium (which would transfer genes in the T-DNA into all the cells). Subsequently these cells should regenerate into whole plants containing the foreign DNA in every cell. The following chapters (3,4) deal with the regeneration systems I have developed for Rubus spp. (Chp.3) and Ribes, Vaccinium and Fragaria spp. (Chp. 4).

Chapter 3.

The regeneration of Rubus spp.

The regeneration of Rubus spp.

Introduction

The incorporation of exogenous DNA into crops using the Agrobacterium vector system, depends upon the ability to successfully regenerate whole plants, from the single cell or few cells into which DNA has been introduced. Inoculation of whole plants is of no use, as only the cells at the site of infection will contain the foreign DNA inserted by the bacterium.

The ability to regenerate a single cell to form a whole plant, known as totipotency, is due to every plant cell and tissue type within a plant having the same complement of genetic information. Every cell of a higher plant originates by mitosis from the single cell of the newly formed zygote, further divisions of which will produce at the completion of its development, differentiated root and shoot apical meristems. The information for plant development is held in the chromosomal DNA of the nucleus, accurate copies of which are made for daughter cells during mitosis. Each cell, therefore, must contain all the information for the production of a whole plant.

If a small explant consisting of only a few cells can be induced to divide and regenerate into a whole plant after infection with Agrobacterium, then there is a strong possibility that this plantlet will have arisen from one or a few transformed cells and hence the resulting plant will contain the foreign DNA in every

cell. Regeneration from one cell is desirable since plantlets regenerated from more than one cell may contain both transformed and non-transformed cells.

A plantlet regeneration technique is therefore essential in the development of an Agrobacterium mediated vector system.

For soft fruit plants, a regeneration system had to be developed before transformation could be attempted, as no suitable system existed. There have been only two reports of whole plant regeneration from Rubus spp. Fiola et al. (1990) used excised cotyledons which have the disadvantage of being time-consuming to extract from a hard endocarp. Also non-dormant seeds are not always available and they represent a segregating generation from heterozygous material which are unlikely to give rise to plants of cultivar potential. The other report by Hall et al. (1986) concerned the production of only three regenerants, two of which died at an early stage. The paper also reported that the plant material was meristematic and, therefore, regeneration was not occurring from somatic cells. Agrobacterium-mediated gene transfer is unlikely to be successful in this instance, as gene transfer would have to occur into every cell of the meristematic region to allow the production of a transformed plant.

In the development of a regeneration system, a number of factors must be considered.

First, the source of explant is important. Generally any healthy tissue can be used for growth and in vitro

plant regeneration, provided it is free of any contaminating microorganisms which compete adversely with the plant material.

Second the growth medium must be considered. It must provide the cells with everything required for growth and development. All growth media are based on a mixture of mineral salts combining macronutrients (N, P, K, Ca, Mg and S) essential for growth of higher plants, and micronutrients (Fe, Mn, Zn, B, Cu, Co and Mo), components of plant cell proteins of metabolic and physiological importance, together with a carbon source (in the form of a sugar) which is important for both respiration and osmotic potential. A chelating agent is usually added to allow insoluble metal ions to be kept in solution. Although vitamins (required by plants to perform catalytic roles in metabolism) are often incorporated into growth media, the actual number and amount varies considerably depending on the explant type and species of plant material used. A solidifying agent is nearly always included, though liquid culture also has applications in tissue culture e.g. suspension cultures (Steward et al. 1952) for the isolation of protoplasts (Evans and Cocking 1973). The solidifying agent is usually agar, which has several useful properties making it ideal for tissue culture. These properties include a setting temperature of 45°C, the fact that it is not digested by plant enzymes and also that it does not react with media constituents.

Plant growth regulators need to be added to the culture medium in most circumstances. These types of substances occur naturally in plant tissues for regulation of growth and development. Synthetic chemicals with similar physiological activities, known as hormones, are generally added to plant tissue cultures to regulate growth and morphogenesis in vitro, rather than for tissue culture nutrition. There are several recognised classes of plant growth substances, though only growth promoters will be considered here. These are as follows:

- auxins
- cytokinins
- gibberellins

The first two classes of compounds are by far the most useful for regulating growth and development in tissue culture.

Auxins control various processes such as cell growth and elongation. They are thought to promote growth by inducing the secretion of hydrogen ions into and through the cell wall. This acidification of the cell wall increases its ability to expand. The electrogenic export of H^+ ions is counteracted by the uptake of potassium ions, thus increasing the osmotic potential of the cell, causing water to enter and make the cell expand. Auxins also control cell growth by affecting protein

synthesis, possibly by modulating transcription (Bevan and Northcote 1981).

The synthetic auxins most commonly incorporated into plant tissue culture media are:

2,4-dichlorophenoxyacetic acid (2,4-D).

indole-3-butyric acid or 4-(indol-3-yl)butyric acid (IBA).

1-naphthylacetic acid (NAA).

Differences occur between auxins in their physiological activity, the extent to which they move within tissues, and whether they are bound within cells or metabolised. Effective concentrations of each auxin will vary, and need to be adjusted for the type of plant material, nature of micropropagation and stage of culture.

The second class of compounds are cytokinins. In tissue culture, cytokinins are necessary for cell division. The mode of action of cytokinins is uncertain. Cytokinins have been shown to activate RNA synthesis and to stimulate protein synthesis and enzyme activity (Kulaeva 1980). Some aminopurines with cytokinin-like activity have been shown to act as reductants in a photochemical reaction with riboflavin, where they are oxidised thereby to adenine (Rothwell and Wright 1967).

Addition of cytokinin to media for callus growth was found to decrease the osmotic potential by increasing the cells turgor potential, the cells becoming less

liable to take up water from the surrounding medium. Auxins have the opposite effect, thus, instead of cell growth, cell elongation occurs.

Thirdly, there are the gibberellins. These are generally not necessary for the induction of growth and differentiation in plant tissue cultures, although gibberellic acid (GA) may become essential for culturing cells at low density (Stuart and Street 1971). GA often produces results similar to those of auxins in encouraging cell growth, possibly due to increased uptake of potassium ions. High concentrations of GA (1-8 mg/l) induce growth of undifferentiated callus cells (Schroeder and Spector 1957, and Gautam et al. 1983). GA has promoted the growth of callus in combination with auxin and low rates of cytokinin. GA alone promoted shoot initiation from Ranunculus scleratus callus, and GA has also acted as a replacement for auxin in the induction of shoot formation from callus (Sekiova and Tanaka 1981).

With suitable nutritional and hormonal conditions in culture, organised growth can be achieved from single cells. Two main mechanisms of plant development have been described, and these are known as organogenesis and embryogenesis (Street 1977).

Organogenesis involves the differentiation of organs from one or a few cells to form roots, shoots and leaves. The process does not depend upon the presence of pre-existing initials. The cells become activated and undergo a series of rapid divisions leading to the

formation of a meristemoid, which gives rise to either a root or a shoot primordium. In Rubus spp. I have found that rapidly growing primordia are not observed for at least 1-2 weeks after the production of explants. The formation of new meristems involves two distinct phases. Dedifferentiation of the original explant begins shortly after isolation of the tissue, with an increase in the rate of cell division, and consequently the formation of a mass of undifferentiated cells. Here living cells, even specialised ones such as collenchyma, can revert to an embryoidal status. Secondly, types of specialised cells will again differentiate. The control of organogenesis is under the direction of both auxins and cytokinins. In tobacco, where the process can easily be controlled, a classic example was described by Miller and Skoog (1953), who found that the concentration of added auxin and cytokinin determined whether the cells grew, and the type of growth produced. On a medium containing 2 mg/l auxin and 0.1 mg/l kinetin only callus proliferated, but if the kinetin concentration was lowered leaving a high auxin concentration, roots developed. In contrast a higher concentration of kinetin relative to auxin led to shoot production.

The most frequent type of regeneration from cultures is root formation. Roots were first observed by Nobecourt (1938) in cultures from carrots and have since been reported for many other tissues, including woody plants (Jacquiot 1951). Roots have been observed to form on Rubus spp. leaf tissue when left in sterile distilled

water (Graham and Williams 1988). Shoots are observed less frequently and are more difficult to induce.

With embryogenesis, the process again starts from undifferentiated cells, similar to organogenesis, by the formation of a mass of dividing cells called an embryoid. This gives rise to the globular, heart-shaped and finally torpedo stages of embryogenesis. The induction of embryos in somatic cell cultures is an excellent example of totipotency i.e. the ability of somatic plant cells, containing all of the information necessary for whole plant production, to become activated and undergo cell division and subsequently regenerate into whole plants.

Fourthly, the physical environment is important. This should be optimal for plant growth. Considerations must be given to temperature, humidity, light intensity and also the culture vessel used. Temperatures adopted for in vitro micropropagation are generally higher than those which the plant would experience in vivo. The temperature adopted for a given species will often vary from experiment to experiment with only slight effects on rate of culture growth. However explant establishment, culture growth, plantlet development and morphogenesis can be temperature dependent. In Vitis spp., leaf blade explants survived in culture at 20°C and 25°C, but growth was not achieved until the temperature was raised to 29°C (Favre 1977). In Brassica campestris, adventitious shoot formation from leaf discs was better at 20°C than at 25°C (Dunwell 1981). Humidity

is also important, with a moist atmosphere required to prevent drying out of tissue cultures.

Light for photosynthesis and photomorphogenesis is required by plants. Both photosynthesis and photomorphogenesis are facilitated by pigments in the tissue which absorb radiation of a particular wavelength. Growth of micropropagated cultures is generally carried out under artificial lighting supplied from fluorescent tubes.

In the development of a regeneration system all the factors discussed must be considered together to determine the optimal conditions for plant regeneration.

Materials and Methods.

Plant Genotypes

- 1) Red raspberries
cv. Autumn Bliss and SCRI selection 8242E6
- 2) Rubus hybrids (red raspberry x blackberry hybrids) cv. Tayberry, Tummelberry and Sunberry.
- 3) Blackberry
cv. Loch Ness.

Maintenance of plant genotypes

Plant genotypes were maintained in vitro on agar solidified (7 g/l) media based on Murashige and Skoog salts containing the following:-

Raspberry multiplication medium (RM)

	mg/l
NH_4NO_3	1650.0
KNO_3	1900.0
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440.0
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370.0
KH_2PO_4	170.0
FeNa.EDTA	36.70
H_3BO_3	6.2
$\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$	22.30
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.60
KI	0.83
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025
Activated charcoal	20000.0.
Biotin	0.01
Calcium pantothenate	1.0
Cysteine-HCl	1.0
Gibberellic acid	2.5
Glutamine	200.0
Glycine	4.0
Inositol	200.0
Nicotinic acid	1.5
Pyridoxine-HCl	1.5
Sucrose	30000.0
Thiamine-HCl	1.1

This medium was adapted from that of Carrie et al 1979

Micropropagation of woody plants.

All woody plant material used in the experiments was introduced into tissue culture as excised buds. These buds formed whole plants within approximately 4 weeks, by which time the single plant produced could be divided into 2-3 explants and used to initiate a tissue culture stock of the plant material. After a further 3-5 weeks each of the resulting plants could be further divided into 4 plants. Raspberry and blueberry plants grow as single shoots whereas the blackcurrants and strawberries grow as a clump of small plants produced on base tissue. For particular experiments where large and abundant leaf tissue was required, both the blackcurrants and the strawberries could be induced to grow as single shoots by reducing the amount of BAP contained within the multiplication medium.

The Rubus tissue, unlike the other three woody species had to be maintained on charcoal containing medium, to absorb the high levels of phenolic compounds produced by wounded tissue.

All explant material for experiments was obtained from cultures maintained at 20°C under warm white fluorescent tubes at 70 $\mu\text{mol m}^{-2}\text{s}^{-1}$, in a 16 hour light:8 hour dark daily regime. All stock cultures were subcultured at 5 week intervals. For a given experiment all explants were derived from plantlets subcultured on the same date. All in vitro plantlets were kept sterile, by culturing aseptically in a lamina flow cabinet. (Diagrams of the material can be seen in the appendix.)

Constituents of media RM and derivatives A and NA.

	mg/l		
	RM	A	NA
NH ₄ NO ₃	1650.0	1650.0	1650.0
KNO ₃	1900.0	1900.0	1900.0
CaCl ₂ .2H ₂ O	440.0	440.0	440.0
MgSO ₄ .7H ₂ O	370.0	370.0	370.0
KH ₂ PO ₄	170.0	170.0	170.0
FeNa.EDTA	36.70	36.70	36.70
H ₃ BO ₃	6.20	6.20	6.20
MnSO ₄ .4H ₂ O	22.3	22.3	22.3
ZnSO ₄ .4H ₂ O	8.6	8.6	8.6
KI	0.83	0.83	0.83
NaMoO ₄ .2H ₂ O	0.25	0.25	0.25
CuSO ₄ .5H ₂ O	0.025	0.025	0.025
CoCl ₂ .6H ₂ O	0.025	0.025	0.025
Agar	7000.0	7000.0	7000.0
Biotin	0.01	-	-
Calcium Pantothenate	1.0	-	-
Cysteine	1.0	-	-
Glutamine	200.0	-	-
Glycine	4.0	2.0	2.0
Inositol	200.0	200.0	100.0
Nicotinic acid	1.5	0.5	0.5
Pyridoxine-HCl	1.5	0.5	0.5
Sucrose	30000.0	20000.0	20000.0
Thiamine-HCl	1.1	0.2	0.1

Other Media Used

Woody plant medium (WPM)

NH ₄ NO ₃	400.0
KNO ₃	-
Ca(NO ₃) ₂ .4H ₂ O	556.0
CaCl ₂ .2H ₂ O	-
K ₂ SO ₄	990.0
MgSO ₄ .7H ₂ O	370.0
FeSO ₄ .7H ₂ O	-
KH ₂ PO ₄	170.0
CaCl ₂ .H ₂ O	96.0
MnSO ₄ .H ₂ O	16.9
ZnSO ₄ .7H ₂ O	8.6
CuSO ₄ .5H ₂ O	0.25
KI	-
H ₃ BO ₃	6.2
NaMoO ₄ .2H ₂ O	0.25
FeSO ₄ .7H ₂ O	27.8
Na ₂ .EDTA	37.3
Agar	7000.0
Glycine	2.0
Inositol	100.0
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Sucrose	20000.0
Thiamine-HCl	1.0

Andersons medium

400.0
480.0
-
440.0
-
370.0
55.7
-
440.0
16.9
8.6
0.025
0.30
6.2
0.25
55.7
74.5
7000.0
2.0
100.0
0.5
0.5
20000.0
1.0

Ref: LLOYD and McCOWN 1980

Ref: Anderson 1980

All media were solidified with agar (7 g/l), and the pH adjusted to 5.6 prior to the addition of agar and before autoclaving. The addition of hormones, vitamins and antibiotics occurred after autoclaving, using a sterile millipore filter (0.2 μ m).

Hormones Used

6-Benzylaminopurine (BAP)

2,4-Dichlorophenoxyacetic acid (2,4-D)

Gibberellic acid (GA)

Indole-3-butyric acid (IBA)

Kinetin (K)

Zeatin Riboside (ZR)

General conditions for leaf disc experiments.

Preparation of plant material

Leaf discs were excised from axenic vigorous young microplants on the fourth week after subculture, using a sterile 6 mm diameter cork borer. The mid-vein of the leaf was included in the discs, which were placed with the abaxial surface uppermost on the various experimental media contained within 9 cm diameter vented plastic petri dishes.

Arrangement of leaf discs

Each petri dish contained ten discs, which were evenly spaced around the dish. The petri dishes were

sealed with "Nescofilm" and placed under a 16 hour day:8 hour night regime provided by fluorescent tubes at $70 \mu\text{mol m}^{-2} \text{s}^{-1}$. The dishes were randomly spaced on the light banks at a temperature of 20°C .

General conditions for all internodal segment experiments.

General conditions for internodal segments were as for leaf disc experiments.

Preparation of plant material.

Internodal stem segments ≈ 1 cm long and without any axillary buds were excised from in vitro proliferating plantlets. The outer tissues were removed to expose the cortical tissue, by peeling under a dissecting microscope, and the explants placed into a petri dish containing the media. The most effective medium for the culture of leaf discs, medium NA, was also used for the internodal stem segment experiments. Again ten segments were placed into each petri dish, the dishes being sealed with "Nescofilm" and cultured as before.

Leaf disc experiments

Specific conditions for each experiment.

Experiment 3.1.

Genotype: Tayberry.

Number of discs: 40 discs / 4 treatments / 1 genotype at 10 discs per plate.

Choice of media: Raspberry media (generally used for the maintenance of Rubus stock cultures but without the activated charcoal).

Choice of hormones:

GA 2.5 mg/l

IBA 0.1 mg/l and GA 2.0 mg/l

BAP 1.0 mg/l and GA 2.0 mg/l

BAP 1.0 mg/l

The hormones were chosen for the following reasons: GA, as it had been shown to induce shoot regeneration on its own, (see introduction), GA with both an auxin and cytokinin also had been shown to induce regeneration and fourthly, a cytokinin had been used alone to look at its effect on cell division.

Experimental conditions: Each plate was placed randomly under the lighting conditions described.

Results were recorded after the experiments had run for a period of 12 weeks.

Comment: This first regeneration experiment examined the effect of RM and a range of hormones on leaf discs.

Results Experiment 3.1.

Within 14 days after explanting all leaf discs (40 discs at each of the 4 treatments, 160 in total) had died and no growth had occurred. The media was therefore altered as were the hormone combinations.

Experiment 3.2.

Genotypes: Tayberry
Sunberry
Loch Ness
8242E6

Number of discs: 20 discs / 4 genotypes / 9 treatments at 10 discs per plate.

Choice of Media: Medium A, a simplified version of RM was used (see methods and materials section).

Choice of Hormones: the hormones combinations were as follows:-

mg/l

- 1 BAP 0.2 and 2,4-D 0.2
- 2 BAP 2.5 and IBA 0.05
- 3 GA 2.5
- 4 BAP 1.0 and GA 2.0
- 5 BAP 2.5 and 2,4-D 0.05
- 6 BAP 0.2 and IBA 0.1
- 7 BAP 0.2 and IBA 0.2
- 8 BAP 0.2 and IBA 2.0
- 9 BAP 4.0 and IBA 2.0

A wider variety of hormones comprising a combination of both auxins and cytokinins and again GA on its own were used.

Experimental conditions: As exp.3.1.

Comment: Medium A with a range of hormone concentrations was examined here.

Results Experiment 3.2.

Callus formation occurred on most of the discs (20 discs of 4 genotypes, 160 in all) at each of the 9 treatments. This was widely variable in both appearance and health, and descriptions follow:

Table 3.2. Amount and description of callus formed on medium A with 9 different hormone combinations.

Hormone comb.	Genotype	No. which died	No. with callus	Type of callus produced
1	Tayberry	1	19	Large amount of hard dark green healthy callus produced.
	Sunberry	0	20	
	8242E6	4	16	
	Loch Ness	3	17	
2	Tayberry	4	16	Light green with dark green areas friable
	Sunberry	3	17	
	8242E6	3	17	
	Loch Ness	8	12	
3	Tayberry	11	9	Small amount of brown callus.
	Sunberry	9	11	
	8242E6	11	9	
	Loch Ness	13	7	
4	Tayberry	10	10	Small amount of unhealth callus
	Sunberry	7	13	
	8242E6	12	8	
	Loch Ness	9	11	
5	Tayberry	4	16	Light green with dark patches highly friable
	Sunberry	1	19	
	8242E6	6	14	
	Loch Ness	3	17	
6	Tayberry	5	15	Slimy green callus
	Sunberry	3	17	
	8242E6	5	15	
	Loch Ness	3	17	
7	Tayberry	4	16	Light green slimy callus
	Sunberry	3	17	
	8242E6	6	14	
	Loch Ness	5	15	
8	Tayberry	4	16	Mainly green and healthy some slimy areas.
	Sunberry	1	19	
	8242E6	6	14	
	Loch Ness	4	16	
9	Tayberry	7	13	Green healthy and variable slimy areas
	Sunberry	6	14	
	8242E6	11	9	
	Loch Ness	8	12	

As can be seen from table 2 on the previous page, the type of callus formed depended upon the hormone combination rather than the genotype. Generally all genotypes produced callus of a similar morphology at the same hormone treatment allowing a general description to be given above.

No plantlet regeneration occurred even after a long period of growth (20 weeks) under any of the treatments. Hormone combination 1 appeared most successful at callus production.

The following figures (3.1-3.2) show callus production from the hormone combinations 0.2 mg/l BAP and 0.2 mg/l 2,4-D (3.1) and 0.2 mg/l BAP and 2 mg/l IBA (3.2) on the blackberry cultivar Loch Ness.

Fig. 3.1

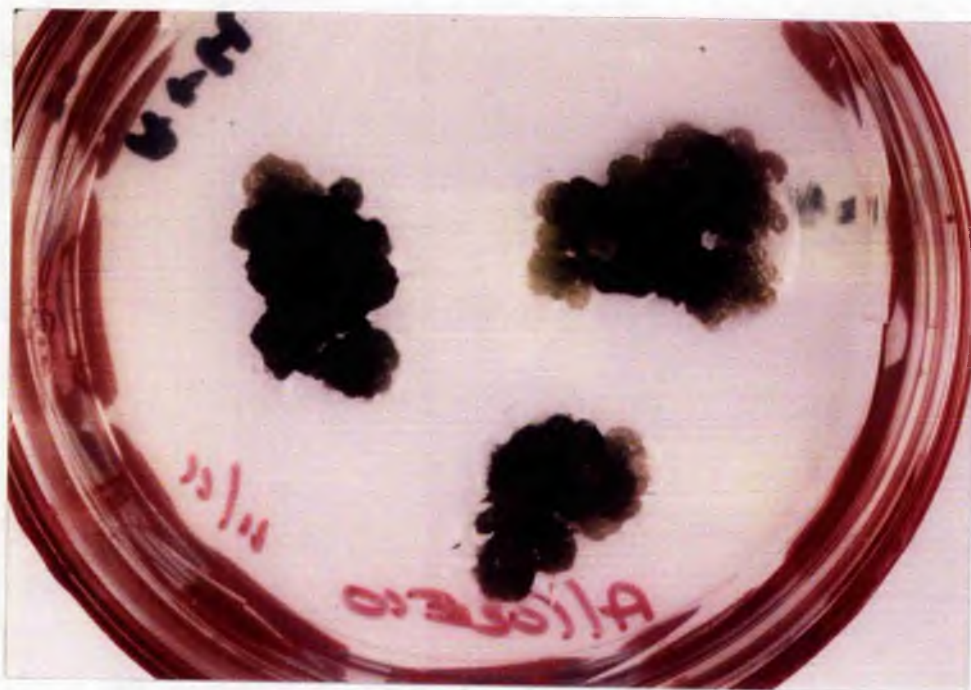


Fig. 3.2



Callus production from leaf discs of the blackberry cv. Loch Ness on media containing the hormone combination BAP 2,4-D (3.1) and BAP and IBA (3.2).

Experiment 3.3.

Genotypes: Tayberry
Sunberry
Loch Ness.

Number of the discs: 20 discs / 3 genotypes / 4 treatments at 10 discs per plate.

Choice of media: A wider range of media was employed; including Andersons medium, woody plant medium, RM and A. RM, Andersons and WPM have all been used in the tissue culture of soft fruit. A proved successful for callus production.

Choice of hormones: BAP at 0.2 mg/l and 2,4-D at 0.2 mg/l. This combination of hormones was chosen as it had proved most successful at callus production in experiment 3.2.

Experimental conditions: As exp.3.1

Comment: A wider range of media and the most successful hormone combination from exp.3.2 were examined here.

Results Experiment 3.3.

Callus production was achieved on 3 of the 4 media (20 discs of each of the 3 genotypes under the 4

treatments, 240 discs in total). The hormone combination of 0.2 mg/l BAP and 0.2 mg/l 2,4-D, which had given the best callus so far (exp 3.2) still failed to induce plantlet regeneration. All 60 discs on Andersons medium died without producing callus.

Table 3.3. Amount and description of callus produced by leaf discs on 4 different media with the hormone combination 0.2 mg/l BAP and 0.2 mg/l 2,4-D.

Media	Genotype	Died	Callused	Type of callus produced
Andersons	Tayberry	20	0	None
	Sunberry	20	0	
	Loch Ness	20	0	
WPM	Tayberry	4	16	Moderate production
	Sunberry	2	18	
	Loch Ness	6	14	
RM	Tayberry	14	6	Small amount of unhealthy callus produced
	Sunberry	12	8	
	Loch Ness	15	5	
A	Tayberry	3	17	Large amount of healthy callus
	Sunberry	0	20	
	Loch Ness	5	15	

Medium A was the most successful for producing callus (see results). This medium was further simplified to produce medium NA and the two were compared in experiment 3.4.

Experiment 3.4.

Genotypes: Tayberry
Sunberry
Loch Ness
8242E6

Number of discs: 20 discs / 4 genotypes / 2 treatments at 10 discs per plate.

Choice of media: Media A and NA were compared

Choice of hormones: Hormone combination 0.2 mg/l BAP and 0.2 mg/l 2,4-D.

Experimental conditions: As exp.1

Comment: Medium A and a simpler version NA were examined here.

Results Experiment 3.4.

Callus production was achieved on both media, however the simpler one, NA, was the most successful giving more profuse and healthy callus than on medium A (there were 20 discs per 4 genotypes and 2 treatments).

Table 3.4. Comparison of the amount and description of callus produced by leaf discs on media A and NA.

Media	Genotype	Died	Callused
A	Tayberry	4	16
	Sunberry	2	18
	Loch Ness	5	15
	8242E6	8	12
NA	Tayberry	2	18
	Sunberry	0	20
	Loch Ness	3	17
	8242E6	5	15

The results obtained from this experiment suggest that medium NA was superior at sustaining cell division, and it was subsequently used in all further experiments.

Experiment 3.5.

Genotype: Sunberry (this had shown the greatest potential for cell division of leaf tissue).

Number of discs: 40 discs / 1 genotype / 16 treatments at 10 discs per plate.

Choice of Media: Media NA

Choice of hormones:

	IBA mg/l			
	0.05	0.1	0.2	2.0
BAP	0.2	0.2	0.2	0.2
mg/l	2.0	2.0	2.0	2.0
	2.5	2.5	2.5	2.5
	4.0	4.0	4.0	4.0

Experimental conditions: As exp.3.1

Comment: Medium NA with a range of IBA and BAP concentrations were examined for potential to induce plantlet regeneration. IBA was substituted for 2,4-D which appeared very effective at callus production but not shoot regeneration.

Results Experiment 3.5.

In this experiment, for the first time plantlet regeneration occurred from the genotype Sunberry. This only occurred, however, on media NA containing 4 of the 16 hormone combinations used, but was achieved successfully at only one of the 16 combinations, this being 0.1 mg/l IBA and 2 mg/l BAP.

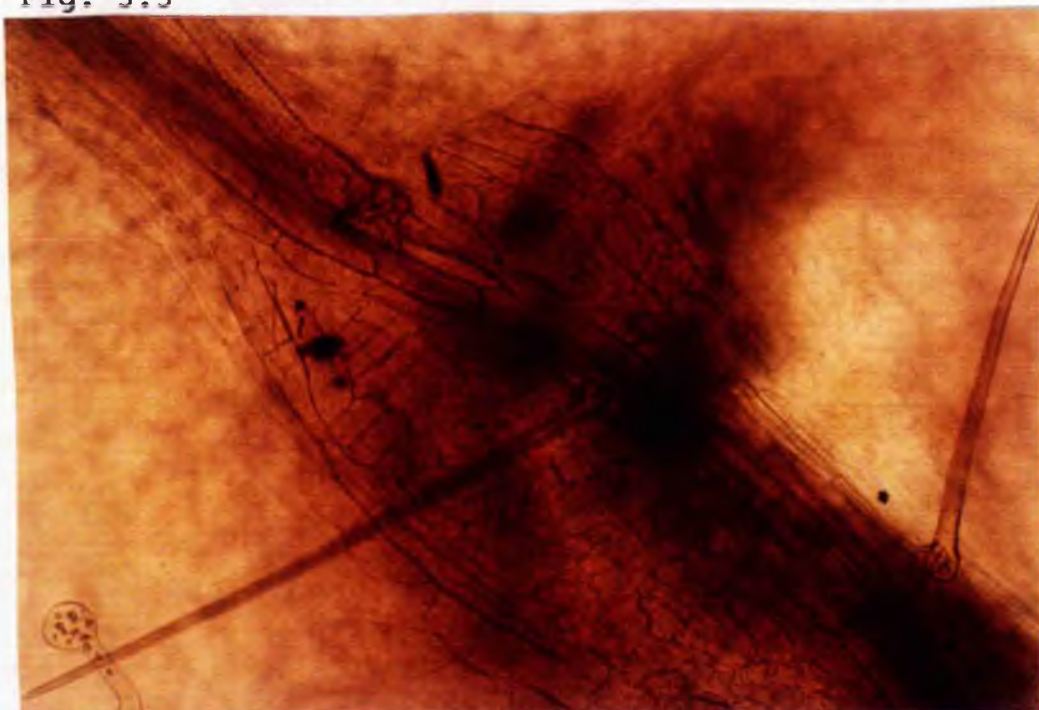
Table 3.5. Comparison of callus production and regeneration potential of the 4 hormone combinations which did induce regeneration.

Hormone combination	Regeneration	%	Callus only
A) 0.1 mg/l IBA and 2 mg/l BAP	18/40	45	16
B) 0.05 mg/l IBA 2.5 mg/l BAP	1/40	2.5	22
c) 0.05 mg/l IBA and 2 mg/l BAP	1/40	2.5	29
D) 0.1 mg/l IBA and 2.5 mg/l BAP	1/40	2.5	28

Regeneration in this experiment was observed to be occurring mainly along the mid-vein, with further regeneration occurring to a lesser extent, and at a later stage along the cut outer surface of the leaf discs.

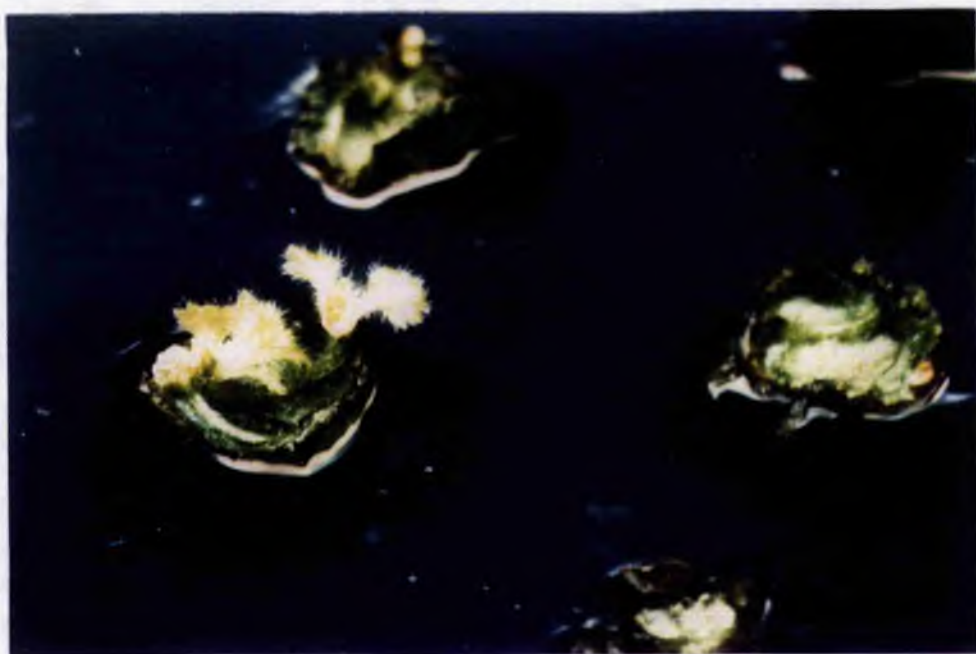
The figures on the following page, show cell division occurring along the mid-vein (3.3) of leaf tissue of the hybridberry Sunberry, leading to plantlet formation (3.4). Callus production can be observed along the cut outer surface of the leaf.

Fig. 3.3



Distortion along mid-vein of a leaf disc of the hybrid berry Sunberry due to cell division.

Fig. 3.4



Callus production and plantlet formation on leaf discs of the hybrid berry Sunberry on media NA with the hormone combination IBA 0.1 mg/l and BAP 2 mg/l.

Experiment 3.6.

Genotype: Sunberry

Number of discs: 140 discs / 1 genotype / 2
treatments at 10 discs per plate.

Choice of Media: NA

Choice of hormones: 0.1 mg/l IBA and 2 mg/l BAP

Experimental conditions: As exp.3.1

Comment: This experiment was carried out to examine
the importance of incorporating the mid-vein in the
discs.

Results experiment 3.6.

The importance of regeneration occurring along the
mid-vein was examined and found to greatly contribute to
the regeneration potential of the discs (140 discs, 1
genotype, 2 treatments).

**Table 3.6. Comparison of callus production and
regeneration potential of leaf discs with and without
the mid-vein.**

	Mid-vein	No mid-vein
No. discs	140	140
Callus	76	57
No. regn.	62	4
% regn.	44	3

The percentage regeneration from leaf discs containing the mid-vein in exps. 3.5 and 3.6 were found to be almost identical.

Experiment 3.7.

Genotype: Sunberry
Tayberry
Loch Ness
Autumn Bliss
8242E6.

Number of discs: 80 discs / 5 genotype / 1 treatment at 10 discs per plate.

Choice of media: NA

Choice of hormones: 0.1 mg/l IBA and 2 mg/l BAP

Experimental conditions: As exp.3.1

Comment: Regeneration potential of a range of plant genotypes was examined here.

Results Experiment 3.7.

This experiment was a repeat of experiment 3.6, using a wider range of plant genotypes including again Sunberry, to ensure that regeneration was not genotype-specific.

Regeneration was extended to all plant genotypes tested, with varying levels of success. The specificity of conditions required by each cultivar, was demonstrated by variation in the level of plantlet regeneration.

(80 discs per 5 genotypes per 1 treatment)

Table 3.7. Comparison of regeneration from 5 different genotypes of Rubus spp.

Plant material	No. discs	Callus	Regeneration	%
Sunberry	80	65	37	46
Loch Ness	80	50	23	29
Tayberry	80	62	35	44
8242E6	80	57	23	29
Autumn Bliss	80	27	7	9

Using chi-squared, results from this experiment suggested that there were significant differences ($P < 0.01$) between the genotypes in their ability to regenerate. Sunberry is the best at regenerating, Autumn Bliss is the poorest.

In this and all the previous leaf disc experiments, leaf discs had been placed abaxial surface uppermost. The next experiment examines the effect of leaf disc orientation on plantlet regeneration.

Experiment 3.8

Genotype: Sunberry

Number of discs: 60 discs/ 1 genotype / 2
treatments / 10 discs per plate.

Choice of media: NA

Choice of hormones: 0.1 mg/l IBA and 2 mg/l BAP

Experimental conditions: As exp.3.1

Comment: The importance of leaf disc orientation was examined, 60 discs being placed on the media with their abaxial surface uppermost and 60 discs adaxial surface uppermost.

Results Experiment 3.8.

This study of the effect of leaf disc orientation on the regeneration potential of 120 discs in all showed a significant difference ($P < 0.001$) between those with the abaxial surface uppermost and those with the adaxial surface uppermost.

Table 3.8. Effect of leaf disc orientation on the regeneration potential of Sunberry.

Surface	Number of discs	Regeneration	% regeneration
Uppermost			
Abaxial	60	31	62
Adaxial	60	7	12

Experiment 3.9.

Genotype: Sunberry

Number of discs: 100 discs / 1 genotype / 2
treatments at 10 discs per plate.

Choice of media: Activated charcoal (routinely used
in Rubus micropropagation media) was incorporated
into medium NA.

Choice of hormones: 0.1 mg/l IBA and 2 mg/l BAP

Experimental conditions: As exp.3.1

Comment: In an attempt to improve regeneration,
activated charcoal was added to the medium to
absorb phenolic compounds, produced by the explants
in response to tissue damage and which may have
been inhibitory to cell division.

Results Experiment 3.9.

Table 3.9. The effects of activated charcoal on the regeneration potential of leaf discs.

	Charcoal	No charcoal
No. discs	100	100
No. died	63	20
Callused	14	70
Regenerated	0	51
% Regn.	0	51

As illustrated in table 3.9, plantlet regeneration was totally inhibited in the presence of activated charcoal, though a small amount of callus was produced on 14 of the 100 leaf discs on the charcoal medium (100 discs per 1 genotype per 2 treatments).

Experiment 3.10.

Genotypes: Sunberry

Tayberry

Loch Ness

Autumn Bliss

8242E6.

Number of discs: 30 discs / 5 genotypes / 5 treatments at 10 discs per plate.

Choice of media: NA, containing 5 levels of ascorbic acid. The levels were as follows: 0, 0.2, 2.0, 20 and 200 mg/l

Choice of hormones: 0.1 mg/l IBA and 2 mg/l BAP.

Experimental conditions: As exp.3.1

Comment: The anti-oxidant ascorbic acid was used in this experiment in an attempt to improve the number of discs regenerating.

Results Experiment 3.10.

The anti-oxidant ascorbic acid was added to the medium in an attempt to increase the regeneration potential of the leaves. This however had widely variable results as indicated in the table on the following page.

Using Genstat, a binomial model was fitted to the variate "number regenerating", using as factors "genotype" and "ascorbic acid". The results of this analysis showed the genotype to be highly significant ($P < 0.001$) in affecting regeneration whereas the concentration of ascorbic acid was not found to be significant ($P > 0.95$).

Table 3.10. The effect of ascorbic acid on the regeneration potential of leaf discs.

Ascorbic acid concentration	No.discs	Callus	No.regn.	% regn.
Autumn Bliss				
0	30	11	6	20
0.2	30	9	3	10
2.0	30	7	3	10
20	30	9	3	10
200	30	5	3	10
Sunberry				
0	30	17	15	50
0.2	30	15	15	50
2.0	30	16	14	47
20	30	12	10	33
200	30	18	17	57
8242E6				
0	30	14	9	30
0.2	30	10	3	10
2.0	30	11	7	23
20	30	9	6	20
20	30	12	6	20
Tayberry				
0	30	17	12	40
0.2	30	9	6	20
2.0	30	16	6	20
20	30	11	4	13
200	30	12	7	23
Loch Ness				
0	30	11	8	27
0.2	30	13	7	23
2.0	30	17	14	47
20	30	16	11	37
200	30	9	6	20

(30 discs 5 genotypes 5 treatments)

Experiment 3.11.

Genotype: Sunberry

Number of the discs: 60 discs / 1 genotype / 2 treatments at 10 discs per plate.

Choice of media: NA

Choice of hormones: 0.1 mg/l IBA and 2 mg/l BAP.

Experimental conditions: For 60 of the discs the conditions were as exp.1. The remaining discs were placed under the same conditions as exp.3.1 except the regime was now one of 24 hour darkness.

Comment: This experiment was set up to examine the importance of light in the regeneration process.

Results Experiment 3.11.

The importance of light in the regeneration process of plant material was examined in this experiment by growing Sunberry leaf discs under conditions of either light or dark.

Table 3.11. Effect of light or dark on leaf disc regeneration.

	No.discs	Callus	No.regn.	% regn.
Light	60	22	38	63
Dark	60	47	0	0

In the dark no regeneration occurred however most of the discs (47/60) produced white callus.

Experiment 3.12.

Genotype: Sunberry

Number of discs: 25 discs / 1 genotype / 1 treatment at 5 discs per plate.

Choice of media: NA

Choice of hormones: 0.1 mg/l IBA and 2 mg/l BAP

Experimental conditions: As exp.3.1

Comment: This experiment was set up to examine the productivity of the leaf disc regeneration procedure rather than the number of discs regenerating.

Results Experiment 3.12.

Productivity was examined in this experiment from 25 discs to give an indication of how effective the regeneration process was. The number of plantlets produced from 25 discs was counted after 4 weeks, 12 weeks and 20 weeks as shown in the tables which follow.

Of the 25 discs, 8 failed to regenerate at all giving an overall % regeneration of 68%.

Table 3.12a. Number of plantlets produced over time from 25 leaf discs. No. discs regenerating are in brackets.

No. of discs	Plantlets 4 weeks	12 weeks	20 weeks
25	33 (14)	92 (17)	511 (17)

Table 3.12b. Average productivity per explant (from 25 discs) at 4, 12 and 20 weeks growth.

Average Productivity	4 weeks	12 weeks	20 weeks
	1.32	3.68	20.44

Table 3.12c. Number of plantlets produced from each of 25 leaf discs.

Disc No.	4 weeks	12 weeks	20 weeks
1	0	0	0
2	1	3	14
3	3	5	42
4	0	0	0
5	3	5	39
6	0	3	19
7	3	5	12
8	1	4	19
9	2	8	52
10	2	5	19
11	4	6	41
12	3	5	30
13	0	0	0
14	0	0	0
15	3	5	12
16	1	7	92
17	4	9	42
18	0	0	0
19	0	0	0
20	0	6	26
21	2	7	15
22	0	0	0
23	1	3	7
24	0	6	30
25	0	0	0

The results on the previous page illustrate clearly an increase in the number of plants being produced over time. The number of discs regenerating increased from four to twelve weeks, after which time however no further discs regenerated.

The following figure (3.5) shows the productivity of plantlet production on one leaf disc of the hybridberry Sunberry. A total of ninety two plantlets were obtained from this one disc by 20 weeks of culture.

Fig. 3.5



Plantlet production from one leaf disc of the hybrid berry Sunberry. In total 90 plantlets were produced from this one disc.

Internodal Segment experiments.

Specific conditions for each experiment.

Experiment 3.13.

Genotypes: Tayberry, Loch Ness and 8242E6

Number of segments: 20 segments / 3 genotypes / 2
treatments 10 segments per plate.

Choice of media: NA

Choice of hormones: 0.2 mg/l BAP and 0.2 mg/l
2,4-D.

0.1 mg/l IBA and 2 mg/l BAP

Experimental conditions: As exp.3.1

Comment: Segments were peeled and placed onto NA
with the above hormone combinations.

Results Experiment 3.13.

Regeneration occurred with all 3 genotypes to a
varying degree and was both genotype and hormone
dependent.

The hormone combination 0.2 mg/l BAP and 0.2 mg/l
2,4-D proved to be more efficient at inducing plantlet
regeneration.

Table 3.13a. Regeneration potential of internodal segments from 3 genotypes of *Rubus* at the hormone combination 0.2 mg/l BAP and 0.2 mg/l 2,4-D.

Plant material	No. segs.	No. with regn.	% regn.
Tayberry	20	15	75
Loch Ness	20	10	50
8242E6	20	10	50

Table 3.13b. Regeneration potential of internodal segments from 3 genotypes of *Rubus* at the hormone combination 0.1 mg/l IBA and 2 mg/l BAP.

Plant material	No. segs.	No. with regn.	% regn
Tayberry	20	9	45
Loch Ness	20	7	35
8242E6	20	5	25

Experiment 3.14.

Genotypes: Tayberry, Loch Ness and 8242E6.

No. of segments: 25 segments / 5 genotypes / 3 treatments at 10 segments per plate.

Choice of media: NA

Choice of hormones: 0.2 mg/l BAP and 0.2 mg/l
2,4-D

Experimental conditions: As exp.3.1

Comment: Peeled segments, non peeled segments and the peelings from segments of Tayberry, Loch Ness

and 8242E6 were placed under the conditions described above to examine what part of the stem tissue would regenerate.

Results Experiment 3.14.

Table 3.14. Comparison of regeneration from peeled and non peeled segments and the peelings.

Plant material	Regeneration from 25 segments					
	Peeled	%	Non peeled	%	Peelings	%
Tayberry	17	68	6	24	0	0
Loch Ness	16	64	5	20	0	0
8242E6	16	64	4	16	0	0

No significant difference occurred between genotypes in the peeled, unpeeled and peeling categories. With regard to the comparison between peeled and unpeeled segments and the peelings, it is clear from the results that no regeneration is likely to come from peelings; there is however a difference between the regeneration ability of peeled and unpeeled segments.

Experiment 3.15.

Genotype: Tayberry, Sunberry, Tummelberry,
8242E6 and Loch Ness.

Number of segments: 25 segments / 5 genotypes /
1 treatment.

Choice of media: NA

Choice of hormones: 0.2 mg/l BAP and 0.2 mg/l 2,4-D

Experimental conditions: As exp.3.1

Comment: The regeneration potential of a wider range of genotypes was examined.

Results Experiment 3.15.

The regeneration potential from segments of a wider range of plant genotypes was examined and regeneration was found to occur from them all.

Table 3.15. Comparison of regeneration from segments from 5 genotypes of Rubus spp.

Genotype	No.segs.	No. regn.	% regn.
Loch Ness	25	16	64
8242E6	25	16	64
Tayberry	25	17	68
Tummelberry	25	12	48
Sunberry	25	14	56

(25 segments per 5 genotypes per 1 treatment)

Experiment 3.16.

Genotype: Tayberry, Loch Ness and 8242E6

Number of segments: 25 segments / 3 genotypes
/ 5 treatments at 10 segments per plate.

Choice of media: NA.

Choice of hormones: Kinetin at 2 mg/l with 2,4-D at 0.2, 1.0, 1.5, 2.0 and 3.0 mg/l.

Experimental conditions: As exp.3.1

Comment: This experiment examined the effects of the potent cytokinin, kinetin.

Results Experiment 3.16.

A few plantlets regenerated with each genotype at 0.2 mg/l 2,4-D and 2 mg/l kinetin. With Tayberry one plantlet also regenerated at 3 mg/l 2,4-D and 2 mg/l kinetin.

Table 3.16. The effect of 2 mg/l Kinetin and various 2,4-D concentrations on regeneration from segments.

2,4-D conc.	No. segs.	No. regenerating		
		Tayberry	8242E6	Loch Ness
0.2	15	2	1	2
1.0	15	0	0	0
1.5	15	0	0	0
2.0	15	0	0	0
3.0	15	1	0	0

Experiment 3.17.

Genotype: Tayberry, Tummelberry, Sunberry, 8242E6 and Loch Ness.

Number of segments: 25 segments / 5 genotypes / 1 treatment at 10 segments per plate.

Choice of media: NA

Choice of hormones: 0.1 mg/l 2,4-D and 2 mg/l ZR

Experimental conditions: As exp.3.1

Comment: The effects of the strong cytokinin zeatin riboside were examined.

Results Experiment 3.17.

With the hormone combination 0.1 mg/l 2,4-D and 2 mg/l zeatin riboside, regeneration occurred from all genotypes.

Table 3.17. Regeneration potential of 5 different genotypes on medium containing the hormones 0.1 mg/l 2,4-D and 2 mg/l ZR.

Plant material	No. segs.	No. regn.	% regn.
8242E6	25	10	40
Loch Ness	25	9	36
Tayberry	25	4	16
Tummelberry	25	7	28
Sunberry	25	4	16

(25 segments per 5 genotypes per 5 treatments)

Experiment 3.18.

Genotype: Tayberry.

No. of segments: 15 segments/ 1 genotype/ 1 treatment at 10 segments per plate.

Choice of media: NA.

Choice of hormones: As exp.3.15

Experimental conditions: As exp.3.1

Comment: The productivity of the internodal segment system was examined here.

Results Experiment 3.18.

Regeneration occurred from 13 of the 15 segments examined (86%), with the number of plantlets produced per segment increasing over time.

Table 3.18a. Number of plantlets produced over time from 15 segments. The number of segments regenerating are in brackets.

No. of segs.	Plantlets 4 weeks	12 weeks	20 weeks
15	20 (10)	48 (13)	87 (13)

Table 3.18b. Average productivity (from 15 segments) at 4, 12 and 20 weeks.

Average productivity	4 weeks	12 weeks	20 weeks
	1.33	3.2	5.87

Table 3.18c. Number of plantlets produced from each of 15 segments.

Segment no.	4 weeks	12 weeks	20 weeks
1	0	0	0
2	2	2	6
3	2	4	4
4	0	4	8
5	2	6	9
6	2	6	14
7	3	3	3
8	1	2	2
9	3	4	7
10	0	0	0
11	3	5	10
12	0	3	9
13	1	2	3
14	1	3	4
15	0	4	9

As in the leaf disc regeneration experiments, the number of plantlets produced increased over time. Again, the number of explants regenerating increased from four to twelve weeks, after which time no further increase occurred.

Although a larger number of internodal segments than leaf discs regenerated, the productivity of internodal segment regeneration is much lower.

Discussion

In the development of a regeneration technique for Rubus spp., the first medium used was that which was already in routine use for raspberry micropropagation (RM). Four different hormone combinations were incorporated into this medium, but it was incapable of sustaining growth and cell division of the leaf discs, which all died by the 14th day of culture (exp.3.1). It was, therefore, totally unsuitable for either callus production or plantlet regeneration.

Medium A, was derived from RM by reducing the amount of sucrose, inositol, thiamine-HCl, glycine, nicotinic acid and pyridoxine-HCl. Biotin, calcium pantothenate and cystein-HCl were omitted. Medium A was used in exp. 3.2 with 9 different hormone combinations, some of which had previously been used in exp.3.1 with RM. Callus was produced to varying degrees on leaf discs at each hormone combination in this experiment (examples of which are shown in figs. 3.1 and 3.2). However the callus produced varied in both amount and state of health. The hormone combination of 0.2 mg/l BAP and 0.2 mg/l 2,4-D was the most successful in terms of callus producing ability. That callus production occurred on the two hormone combination also used in exp.3.1, demonstrated the importance of the composition of the medium as well as hormone balance.

The importance of media composition (exp.3.3), was examined utilising the hormone combination 0.2 mg/l BAP and 0.2 mg/l 2,4-D (exp.3.2), comparing the media; A, WPM, RM and Andersons. Callus production was most successfully achieved with medium A, followed by WPM and RM. Callus production on RM, was probably due to the hormone 2,4-D, which appeared very effective in inducing callus production. Andersons medium was incapable of sustaining cell growth and division, possibly due to lower nitrogen levels. The success of medium A over the other three media investigated, suggested that a simple M+S salts based medium, would be most effective in the production of callus. Medium A was further simplified to produce medium NA (exp.3.4), which proved more successful than A. The difference between the two media were as follows, media A contained 100 mg/l of inositol more than media NA and 0.1 mg/l more of thiamine-HCl. This showed how specific good growth conditions were.

In experiment 3.5, Sunberry was chosen as the genotype on which to examine the effects of the hormones IBA and BAP, because this genotype produced callus most successfully. The auxin 2,4-D was deliberately not chosen as it was too efficient at causing callus production, which may have been inhibiting regeneration, thus the auxin hormone IBA was used in conjunction with the cytokinin BAP in an experiment designed to identify the best ratio of cytokinin:auxin for regeneration (if any). In this experiment plant regeneration was first achieved (fig 3.4). Only one of the sixteen different

combinations induced organogenesis to any useful level, again implying that the hormone balance is critical.

The formation of plantlets occurred mainly along the mid-vein (exp.3.6). Initiation occurred around the 9th day of culture, with regeneration along the mid-vein visible by the 14th day. Whole plantlets formed by 14-28 days of culture. Regeneration using this media (NA with the hormones 0.1 mg/l IBA and 2 mg/l BAP) was extended to other genotypes (exp.3.7). Sunberry remained the most responsive with 37 out of 80 discs (46%) regenerating, followed by Tayberry with 35 (46%), Loch Ness and 8242E6 with 23 (29%) and finally Autumn Bliss with only 7 discs regenerating (9%). The occurrence of regeneration mainly along the mid-vein suggested that auxin was important as this moves mainly along the mid-rib of the leaf (Avery 1935). When comparisons of regeneration were made on leaf material with and without the mid-vein, large differences in regenerative ability occurred.

Orientation was also important (exp.3.8) with 62% of leaf discs regenerating with the abaxial surface (under surface) uppermost compared with only 12% regeneration occurring when the adaxial surface (upper surface) was uppermost. Two suggestions as to why this occurred were; first, that if regeneration occurred mainly along the mid-vein, then when the leaf is placed with its adaxial surface uppermost then the plantlet would have to push its way to the surface, whereas when placed in the reverse orientation when regeneration occurred the plantlet was already at the surface; Second there could

be a light effect due to shielding of the regeneration surface.

It was feared that phenolic compounds released from the leaf disc due to cell damage occurring during preparation might be responsible for the inhibition of regeneration. Activated charcoal was therefore added to the medium (exp.3.9) in an attempt to prevent any such inhibition. Carrie et al. (1979) advocated the use of activated charcoal to assist in the development of roots, and improve the in vitro growth of red raspberry genotypes that are difficult to culture. This substance, however, completely inhibited organogenesis of leaf discs. Activated charcoal is not a growth regulator but it has been shown to modify the medium by absorbing a wide range of compounds and can therefore give unpredictable results. Constantini (1976) found that 1% activated charcoal nullified the effects of 2 mg/l BAP in the media. The activated charcoal in this experiment was probably absorbing some of the hormones present, even though it was added to the medium after autoclaving and prior to setting. The effect was therefore to alter the auxin/cytokinin balance. This again emphasised the importance of the correct hormone balance to stimulate regeneration.

Inclusion of the anti-oxidant ascorbic acid in the media (exp.3.10) had very variable results. Metabolism of the ascorbic acid by the plant material may explain this. The importance of light in the regeneration process was examined (exp.3.11). No regeneration

occurred under conditions of total darkness, and only white callus was produced due to there being no light to photosynthesise and the medium providing everything the cells required for growth and division.

A productivity study (exp.3.12) was carried out to determine how many plantlets were produced rather than how many discs regenerated. The discs were examined after 4, 12 and 20 weeks. Twenty weeks was the longest that discs could be left on the medium, due to the plantlets being squashed between the lid and base of the dish and once removed, the discs disintegrated and could not be placed into a culture jar intact. An increase in the number of plantlets produced over time was observed, with 30 plantlets produced by week 4, increasing to 511 plantlets produced by week 20. From week 4 to week 12, the number of discs regenerating increased from 14 to 17, after which time no further increase occurred.

Regeneration of internodal segment material occurred on both combinations of hormones used (exp.3.13) and, therefore, was not as specific in terms of the conditions required for initiation of regeneration as leaf disc material was.

The importance of peeling segments was highlighted in experiment 3.14 where the removal of the outer cuticle from stems gave a significant ($P < 0.01$) increase in the number which regenerated. This indicated that meristematic primordia arose beneath the surface layer which is in accord with the findings of Miller and Skoog (1953).

Although a greater percentage of internodal segments than leaf discs regenerated, the productivity overall was greater from leaf discs. On average, 20 plantlets were produced from each leaf disc (exp.3.13) with only 6 plantlets produced from each internodal segment (exp.3.18) after 20 weeks of culture.

Overall the regeneration from leaf discs and internodal segments varied by 20% between experiments.

The development of regeneration techniques in Rubus spp. facilitates the use of genetic manipulation techniques and also studies on somaclonal variation.

The techniques should prove useful to Rubus breeders and commercial firms specialising in the in vitro propagation of Rubus by speeding up the breeding and propagation processes, and allowing the insertion of exogenous DNA into cultivars and breeding material without the co-transfer of undesirable characteristics.

Chapter 4.

The regeneration of Fragaria, Ribes, and Vaccinium spp.

The regeneration of Fragaria, Ribes and Vaccinium spp.

Introduction

As described in chapter 3 (on the regeneration of Rubus spp.), every plant cell has the entire complement of genetic material, and has the potential therefore to be manipulated to form a whole plant. As in Rubus spp., no suitable regeneration systems were available when I began the project, to allow transformation experiments to be carried out on Ribes, Vaccinium and Fragaria spp. This regeneration obstacle again had to be overcome as an initial step in the development of a transformation process.

Materials, Methods and Results.

The materials, methods and results for each of the above species are divided into 3 sections dealing with:-

- 4.1. Regeneration of Fragaria spp.
- 4.2. Regeneration of Ribes spp.
- 4.3. Regeneration of Vaccinium spp.

Section 4.1. The regeneration of Fragaria spp.

Materials and Methods.

Genotypes of Strawberry used.

selection E2a

selection 71WC64

cv. Rapella

Maintenance of plant genotypes.

Plant genotypes were maintained in vitro on the following medium.

Strawberry Multiplication Medium (SM).

NH ₄ NO ₃	1650.0
KNO ₃	1900.0
CaCl ₂ ·2H ₂ O	440.0
MgSO ₄ ·7H ₂ O	370.0
KH ₂ PO ₄	170.0
FeNa.EDTA	36.70
H ₃ BO ₃	6.2
MnSO ₄ ·7H ₂ O	22.30
ZnSO ₄ ·7H ₂ O	8.60
KI	0.83
Na ₂ MoO ₄ ·2H ₂ O	0.25
CuSO ₄ ·5H ₂ O	0.025
CoCl ₂ ·6H ₂ O	0.025
Agar	8000.0
Benzylaminopurine	1.0
Biotin	0.1
Calcium Pantothenate	1.0
Cysteine	1.0
Glycine	2.0
Indolebutyric acid	0.1
Inositol	100.0
Nicotinic Acid	1.50
Pyridoxine-HCl	1.50
Sucrose	20000.0
Thiamine-HCl	1.10

pH 5.6

As in the Rubus regeneration experiments, all plant material was obtained from cultures grown at 20°C under warm white fluorescent tubes at 70 $\mu\text{mol m}^{-2}\text{s}^{-1}$ in a 16 hour light:8 hour dark regime. All stock cultures were subcultured at 5 week intervals. All explant material for any given experiment was derived from plantlets subcultured on the same date. All plant material was sterile and all experiments were carried out aseptically in a lamina flow cabinet.

Constituents of media used in the regeneration experiments.

	Jones medium		Liu medium
		mg/l	
NH ₄ NO ₃	1650.0		1650.0
KNO ₃	1900.0		1900.0
CaCl ₂ .2H ₂ O	440.0		440.0
MgSO ₄ .7H ₂ O	370.0		370.0
KH ₂ PO ₄	170.0		170.0
FeNa.EDTA	36.70		36.70
H ₃ BO ₃	6.2		6.2
MnSO ₄ .7H ₂ O	22.30		22.30
ZnSO ₄ .7H ₂ O	8.60		8.60
KI	0.83		0.83
Na ₂ MoO ₄ .2H ₂ O	0.25		0.25
CuSO ₄ .5H ₂ O	0.025		0.025
CoCl ₂ .6H ₂ O	0.025		0.025
Agar	7000.0		7000.0
Casein hydrolysate	-		600.0
Inositol	100.0		100.0
Sucrose	20000.0		20000.0
Thiamine-HCl	0.1		0.4

M+S salts, Murashige and Skoog (1962).

Jones et al. (1988).

Liu and Sanford (1988).

Other media used

NA medium as described in chapter 3, page 66.

All media were prepared as in the Rubus experiments with the pH adjusted to 5.6 prior to the addition of agar, and before autoclaving. The addition of hormones and vitamins occurred after autoclaving using a sterile millipore filter (0.2 µ).

Hormones Used

6-Benzylaminopurine (BAP)

2,4-Dichlorophenoxyacetic acid (2,4-D)

Indole-3-butyric acid (IBA)

General conditions for strawberry experiments.

Preparation of plant material.

As in the Rubus experiments, leaf discs were excised from young, axenic, vigorous, microplants on the fourth week after subculture, using a sterile 6 mm diameter cork borer. Again the mid-vein was included in the discs which were then placed with their abaxial surface uppermost on the various experimental media, contained within 9 cm diameter petri dishes.

For strawberry bases, a very small piece of plant material (5 mm in length) was removed from the base of the tissue (base tissue) where the plantlets originate (see appendix page 356 for fuller description of base tissue).

Arrangement of the discs and bases.

Each petri dish contained 10 discs (or bases), which were evenly spaced around the dish. The petri dishes were sealed with "Nescofilm" and placed under a 16 hour light:8 hour dark regime, provided by fluorescent tubes at $70 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$. The dishes were randomly spaced on the light banks at a temperature of 20°C .

Leaf disc experiments and results.

Specific conditions for each experiment.

Experiment 4.1.1.

Genotype: Rapella

E2a

71WC64

Number of discs: 20 discs / 3 genotype / 3 treatments.

Choice of media: Strawberry Multiplication Medium (generally used for maintenance of stock cultures).

Choice of hormones:

1. BAP 2.0 mg/l and IBA 0.1 mg/l.
2. BAP 0.2 mg/l and 2,4-D 0.2 mg/l.
3. BAP 2.5 mg/l and IBA 0.5 mg/l.

The above hormone combinations were chosen for the following reasons: The first combination was chosen as it was the commonly used hormone combination for strawberry multiplication. The second combination included the hormone 2,4-D, generally found to induce callus production. The third combination was chosen in an attempt to induce regeneration.

Experimental conditions: Each plate was placed randomly under the conditions described. Results were recorded after the experiment had run for a 12 week period.

Comment: Strawberry multiplication medium was used in this initial regeneration experiment along with 3 hormone combinations.

Results Experiment 4.1.1.

Strawberry multiplication medium proved totally unsuitable for the culture of leaf discs, with the death of most of the discs (172 discs) occurring within 2 weeks.

Table 4.1.1. Amount of callus produced by leaf discs on SMM with 3 different hormone combinations.

Hormone combin.	Genotype	No. discs	Died	Callused	% callus
1	Rapella	20	20	0	0
	E2a	20	19	1	5
	71WC64	20	20	0	0
2	Rapella	20	18	2	10
	E2a	20	20	0	0
	71WC64	20	17	3	15
3	Rapella	20	19	1	5
	E2a	20	19	1	5
	71WC64	20	20	1	5

A small amount of unhealthy yellowish callus was produced on this medium containing the hormone

combination BAP at 0.2 mg/l and 2,4-D at 0.2 mg/l, around the cut surface of only 5 of the 180 discs cultured. The other two hormone combinations produced only a tiny amount of callus on a total of 4 discs. The use of this medium was subsequently abandoned, and medium NA used in the next experiment.

Experiment 4.1.2.

Genotype: Rapella.

Number of discs: 40 discs / 1 genotype / 2 treatments.

Choice of media: Medium NA used for Rubus regeneration.

Choice of hormones:

1. BAP 0.2 mg/l and 2,4-D 0.2 mg/l.
2. BAP 2 mg/l and IBA 0.1 mg/l.

The hormones were chosen as they had been found to be successful in Rubus spp. for callus production (combination 1 and 2) and plantlet regeneration (combination 2),(chapter 3).

Experimental conditions: As exp.4.1.1

Comment: NA was chosen as it had proved successful in regeneration experiments involving Rubus spp.

Results Experiment 4.1.2.

In this experiment, callus production was achieved on medium NA containing both the hormone combinations: 1) 0.2 mg/l BAP with 0.2 mg/l 2,4-D and 2) 0.1 mg/l IBA with 2 mg/l BAP. Callus produced by hormone combination 1 was more green, healthy and profuse than that produced by hormone combination 2. Regeneration was achieved on this medium from the hormone combination 1, on 8 of the 40 discs cultured.

Table 4.1.2. Amount of callus and regeneration produced by the cv Rapella, on medium NA with 2 different hormone combinations.

Hormone combination.	No. of explants	No. with callus	No.regen. shoots.	% shoot prodn.
1	40	20	8	20
2	40	9	0	0

The next experiment went on to examine if this regeneration medium was cultivar-specific and if regeneration would occur under conditions of total darkness.

Experiment 4.1.3.

Genotype: Rapella

E2a

71WC64

Number of discs: 40 discs / 3 genotypes / 2

treatment.

Choice of medium: NA.

Choice of hormones: BAP 0.2 mg/l and 2,4-D 0.2 mg/l.

Experimental conditions: As exp.4.1.1, except half of the discs were maintained under conditions of total darkness.

Comment: Variation in regeneration ability of 3 different cultivars on the same medium was examined in this experiment. The effect of two different light regimes was also examined.

Results Experiment 4.1.3.

In this experiment, regeneration under a light regime was extended to all 3 genotypes on medium NA with the hormones 0.2 mg/l BAP and 0.2 mg/l 2,4-D. Rapella again regenerated, this time 12 of the 40 discs regenerated (30%), compared with 8 (20%) in the previous experiment.

Under conditions of total darkness no shoot regeneration occurred and only white callus was produced. In contrast, shoots and healthy green callus were produced under the light conditions.

Table 4.1.3. Shoot regeneration from 3 strawberry genotypes.

Genotype	Regime	No. of explants	No. with callus	No. regen. shoots	% shoot regen.
Rapella	Light	40	13	12	30
	Dark	40	14	0	0
E2a	Light	40	9	7	17
	Dark	40	7	0	0
71WC64	Light	40	12	14	35
	Dark	40	12	0	0

Experiment 4.1.4.

Genotype: Rapella

E2a

71WC64

Number of discs: 20 discs / 3 genotypes / 1 treatment.

Choice of media: Jones medium. This medium was found by Jones et al. (1988), to induce shoot regeneration from callus of 6 out of 9 strawberry varieties including Rapella, with regeneration varying from 30-50%.

Choice of hormones: BAP 0.2 mg/l and 2,4-D 0.2 mg/l.

Experimental conditions: As exp.4.1.1

Comment: The effect of a medium previously shown to induce shoot regeneration from leaf discs of 3

strawberry cultivars was examined.

Results Experiment 4.1.4.

This experiment used a medium previously shown to induce shoot regeneration. Here, however, as illustrated in table 4.1.4, no shoots regenerated from the healthy callus produced. Root production was achieved on 2 of the genotypes used.

Table 4.1.4. Effect of Jones medium on leaf discs of 3 strawberry genotypes.

Genotype	No. of explants	No. with callus	No. regen. roots	% root regen.
Rapella	20	16	0	0
71WC64	20	18	3	15
E2a	20	16	3	15

The use of this medium was subsequently abandoned and alternatives used.

Experiment 4.1.5.

Genotype: Rapella

E2a

71WC64

Number of discs: 20 discs /3 genotypes / 1 treatment.

Choice of media: Liu medium. Liu and Sanford (1988) found this medium to be successful at regenerating shoots from leaf discs of the

cultivar Allstar.

Choice of hormones: BAP 2.5 mg/l and IBA 0.5 mg/l.

Experimental conditions: As exp.4.1.1

Comment: This medium was found by Liu and Stanford (1988) to induce shoot regeneration on one of two strawberry cultivars (Allstar but not Honeoye).

Results Experiment 4.1.5.

In this experiment callus production was achieved on all 3 genotypes to variable degrees, and a small amount of shoot regeneration (15% for 71WC64 and 10% for E2a) was achieved from callus produced on discs of 2 of the 3 genotypes used.

Table 4.1.5. Effect of Liu's medium on leaf discs of 3 strawberry genotypes.

Genotype	No. of explants	No. with callus	No. regen. shoots	% shoot regen.
Rapella	20	5	0	0
71WC64	20	13	3	15
E2a	20	8	2	10

Although this treatment did induce shoot regeneration from 2 of the 3 cultivars used, the percentage regeneration was low and again an alternative medium was used in the next experiment.

Experiment 4.1.6.

Genotype: Rapella

E2a

71WC64

Number of discs: 20 discs /3 genotypes /1 treatment.

Choice of media: Basic medium. This medium contained only M+S salts, sucrose, agar and hormones.

Choice of hormones: BAP 0.2 mg/l and 2,4-D 0.2 mg/l.

Experimental conditions: As exp.4.1.1

Comment: In this experiment a basic medium comprising M+S salts, sucrose, agar and the hormones BAP at 0.2 mg/l and 2,4-D also at 0.2 mg/l was used to examine its effect on leaf tissue.

Results Experiment 4.1.6.

No shoot production was achieved on this medium; however, the medium induced root regeneration from 1 of the 3 cultivars used. Using the Chi-squared goodness of

fit test, significantly different results ($P < 0.001$) were obtained for callus production on the 3 cultivars.

Table 4.1.6. Effect of a basic medium on leaf discs of 3 strawberry genotypes.

Genotype	No. of explants	No. with callus	No. regen. roots	% root regen.
Rapella	20	20	0	0
71WC64	20	13	4	20
E2a	20	1	0	0

Experiment 4.1.7.

Genotype: Rapella

E2a

71WC64

Number of discs: 40 discs / 3 genotypes / 1 treatment.

Choice of media: SMM with 600 mg/l Casein Hydrolysate.

Choice of hormones: BAP 2.5 mg/l and IBA 0.5 mg/l.

Experimental conditions: As exp.4.1.1

Comment: Casein hydrolysate (CH), found by Liu and Stanford (1988), to be successful in improving regeneration, was incorporated into SMM.

Results Experiment 4.1.7.

The addition of CH, greatly increased the callus producing ability of SMM (which had been almost totally unsuccessful at callus production in exp.4.1.1). Here 80% in total of all discs callused (40 discs of 3 genotypes under 1 treatment) compared with 8% of discs (20 discs of 3 genotypes in SMM under the same hormone combination as used in this experiment) in exp.4.1.1.

Callus production was greatly increased due to the presence of this component which was subsequently used in the following experiment with the hormones 0.2 mg/l BAP and 0.2 mg/l 2,4-D.

Table 4.1.7. Effect of SMM containing casein hydrolysate on 3 strawberry genotypes.

Genotypes	No. of explants	No. with callus	% callus	No. regen.
Rapella	40	38	95	0
71WC64	40	27	68	0
E2a	40	31	78	0

Experiment 4.1.8.

Genotype: Rapella

E2a

71WC64

Number of discs: 40 discs / 3 genotypes / 1 treatment.

Choice of media: SMM with 600 mg/l Casein hydrolysate.

Choice of hormones: BAP 0.2 mg/l and 2,4-D 0.2 mg/l.

Experimental conditions: As exp.4.1.1

Comment: SMM with casein hydrolysate, successful at callus production (exp.4.1.7), was used in this experiment along with the hormone combination 0.2 mg/l BAP and 0.2 mg/l 2,4-D, successful at shoot regeneration in medium NA (exp.4.1.2).

Results Experiment 4.1.8.

Table 4.1.8. Effect of CH in SMM with 0.2 mg/l BAP and 0.2 mg/l 2,4-D.

Genotype	No. explants	No. callus	No. shoots
Rapella	40	34	0
E2a	40	27	0
71WC64	40	31	0

Although SMM with CH was successful at cell growth and proliferation with the hormones IBA and BAP but not shoot production, and the hormone combination BAP and 2,4-D at the above concentrations induced shoots in NA,

when the BAP and 2,4-D combination was used in SMM, this treatment was still unable to produce shoots.

Experiment 4.1.9.

Genotype: Rapella

E2a

71WC64

Number of discs: 40 discs / 3 genotypes / 1 treatment.

Choice of media: NA with 600 mg/l Casein hydrolysate.

Choice of hormones: BAP 0.2 mg/l and 2,4-D 0.2 mg/l.

Experimental conditions: As exp.4.1.1

Comment: NA with casein hydrolysate, successful at callus production (exps.4.1.7 and 4.1.8), was used in this experiment in medium NA along with the hormone combination 0.2 mg/l BAP and 0.2 mg/l 2,4-D, successful at shoot regeneration (exp.4.1.2).

Results Experiment 4.1.9.

Table 4.1.9. Effect of CH in NA with 0.2 mg/l BAP and 0.2 mg/l 2,4-D.

Genotype	No.explants	No.callus	No.shoots	% shoots
Rapella	40	34	2	5
E2a	40	29	1	2.5
71WC64	40	29	4	10

CH, in medium NA, appeared to decrease the amount of explants regenerating shoots. For example, regeneration from medium NA, without CH, from the cv. Rapella was obtained at a level of 20% in exp.4.1.2 and and 30% in exp.4.1.3, compared with 5% in this experiment. This apparent decrease though could be due to variability in regeneration potential, which has been observed in various experiments.

Experiment 4.1.10.

Genotype: Rapella

E2a

71WC64

Number of bases: 30 bases / 3 genotypes / 1 treatment.

Choice of media: SMM and NA

Choice of hormones: BAP 2.5 mg/l and IBA 0.5 mg/l.

BAP 0.2 mg/l and 2,4-D 0.2
mg/l.

Experimental conditions: As exp.4.1.1

Comment: Strawberry base tissue was examined here
for regenerative ability.

Results experiment 4.1.10.

Table 4.1.10. Effect of medium SMM on strawberry base
tissue.

Genotype	No. of explants	No. with callus	No. regen. shoots	% shoot regen.
SMM				
IBA and BAP				
Rapella	30	9	12	40
71WC64	30	12	15	50
E2a	30	7	16	53
SMM				
BAP and 2,4-D				
Rapella	30	14	2	6
71WC64	30	10	5	16
E2a	30	11	2	6
NA				
IBA and BAP				
Rapella	30	7	2	6
71WC64	30	9	4	13
E2a	30	9	1	3
NA				
BAP and 2,4-D				
Rapella	30	7	15	50
71WC64	30	11	9	30
E2a	30	7	12	40

Regeneration was successfully achieved under two of the four treatments from strawberry base tissue from all 3 genotypes. The other two treatments did induce regeneration but to a much lesser extent.

This experiment demonstrates the importance of the correct hormone combination in a particular medium. With the hormone combination IBA and BAP, explant regeneration was most effectively achieved in SMM, whereas with the hormone combination BAP and 2,4-D, explant regeneration was more effective in NA.

Experiment 4.1.11.

Genotype: Rapella

Number of discs: 20 discs / 1 genotype / 1 treatment.

Choice of media: NA.

Choice of hormones: BAP 0.2 mg/l and 2,4-D 0.2 mg/l.

Experimental conditions: As exp.4.1.1

Comment: This experiment examined the productivity of leaf disc regeneration rather than the number of discs regenerating.

Results Experiment 4.1.11.

Table 4.1.11a. Number of plantlets produced over time from 20 leaf discs. No. of discs regenerating are in brackets.

No. discs	4 weeks	12 weeks	20 weeks
20	10 (8)	24 (10)	62 (14)

Table 4.1.11b. Average productivity per explant (from 20 explants) at 4, 12 and 20 weeks.

Average Productivity	4 weeks	12 weeks	20 weeks
	0.5	1.2	3.1

Table 4.1.11c. Number of plantlets produced from 20 individual leaf discs.

Disc number	4 weeks	12 weeks	20 weeks
1	0	0	2
2	0	1	1
3	0	0	0
4	2	4	14
5	0	3	9
6	0	0	3
7	1	1	1
8	0	0	0
9	0	0	0
10	1	2	2
11	0	0	0
12	0	0	0
13	1	1	6
14	0	0	4
15	2	5	11
16	0	0	0
17	0	0	2
18	1	1	1
19	1	3	3
20	1	3	3

In this experiment the number of plantlets produced increased over time, as did the number of discs regenerating.

This experiment, along with experiments 4.1.2 and 4.1.3 (involving the same medium and hormones), demonstrated the variability of results obtained in the regeneration results, with, in this exp., 50% of the discs regenerated after 12 weeks compared with 20% of discs in exp.4.1.2, and 30% in exp.4.1.3.

Experiment 4.1.12.

Genotype: Rapella.

No. of discs: 20 discs / 1 genotype / 1 treatment repeated 5 times.

Choice of media: NA.

Choice of hormones: BAP 0.2 mg/l and 2,4-D 0.2 mg/l.

Experimental conditions: As exp.4.1.1

Comment: This experiment was set up to examine the variability of results obtained during the regeneration experiments. Five replicates of the same experiment were set up with the petri dishes containing the discs, allocated at random to a replicate number. The petri dishes were then placed

at random under the physical conditions described, and left for twelve weeks when the results were recorded.

Results Experiment 4.1.12.

The results of this experiment illustrate the variability in results obtained from regeneration experiments.

Table 4.1.12. Shoot regeneration from 5 replicates of the same experiment.

Replicate no.	No. discs	No. regen.	% regen.
1	20	10	50
2	20	7	35
3	20	12	60
4	20	9	45
5	20	5	25

This experiment shows significant differences ($P < 0.01$) occurring between regeneration, in 5 replicates of the same experiment. In replicate number 1, half of all the discs regenerated compared with replicate 5 where only a quarter of the discs regenerated.

The following figure (4.1) illustrates the regeneration of strawberry cv. Rapella on NA with the hormone combination 0.2 mg/l BAP and 0.2 mg/l 2,4-D.

The plantlets can be seen forming on the mid-vein of the leaf with callus forming around the edges.

at random under the physical conditions described, and left for twelve weeks when the results were recorded.

Results Experiment 4.1.12.

The results of this experiment illustrate the variability in results obtained from regeneration experiments.

Table 4.1.12. Shoot regeneration from 5 replicates of the same experiment.

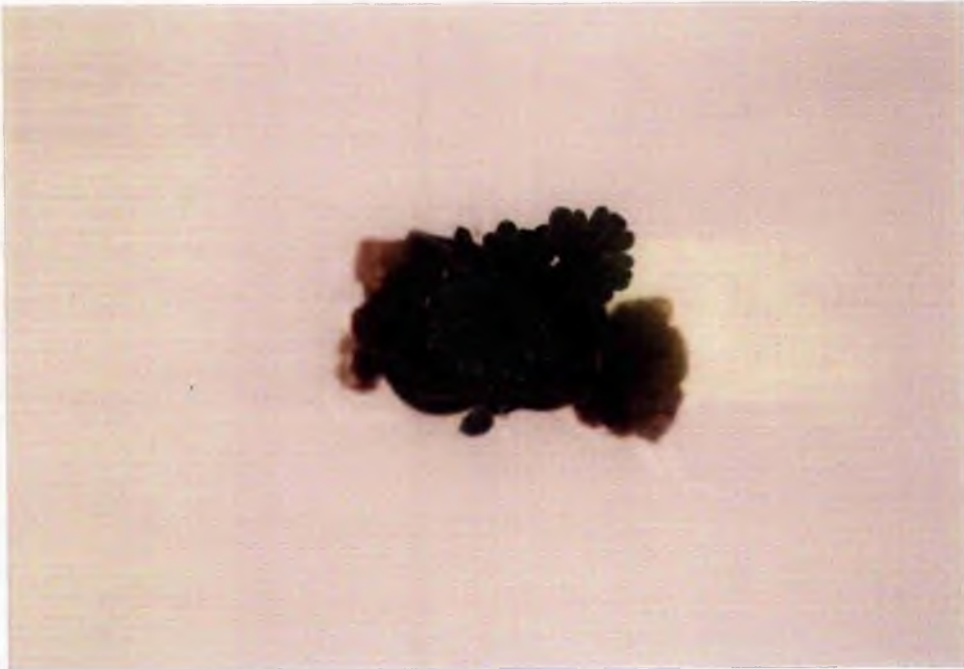
Replicate no.	No. discs	No. regen.	% regen.
1	20	10	50
2	20	7	35
3	20	12	60
4	20	9	45
5	20	5	25

This experiment shows significant differences ($P < 0.01$) occurring between regeneration, in 5 replicates of the same experiment. In replicate number 1, half of all the discs regenerated compared with replicate 5 where only a quarter of the discs regenerated.

The following figure (4.1) illustrates the regeneration of strawberry cv. Rapella on NA with the hormone combination 0.2 mg/l BAP and 0.2 mg/l 2,4-D.

The plantlets can be seen forming on the mid-vein of the leaf with callus forming around the edges.

Fig. 4.1



Plantlet formation on a leaf disc of the strawberry cv. Rapella. Callus formation can be seen occurring along the cut outer surface with regeneration occurring along the mid-vein.

Section 4.2. The regeneration of Ribes spp.

Materials and Methods.

Genotypes of blackcurrant plants used.

cv. Ben More

cv. Baldwin

cv. Jet

Maintenance of plant genotypes.

Plant genotypes were maintained in vitro on the following medium.

Blackcurrant medium (BM).

	mg/l
NH ₄ NO ₃	1650.0
KNO ₃	1900.0
CaCl ₂ .2H ₂ O	440.0
MgSO ₄ .7H ₂ O	370.0
KH ₂ PO ₄	170.0
FeNa.EDTA	36.70
H ₃ BO ₃	6.2
MnSO ₄ .7H ₂ O	22.30
ZnSO ₄ .7H ₂ O	8.60
KI	0.83
Na ₂ MoO ₄ .2H ₂ O	0.25
CuSO ₄ .5H ₂ O	0.025
CoCl ₂ .6H ₂ O	0.025
Agar	7000.0
Benzylaminopurine	1.0
Glycine	2.0
Inositol	200.0
Nicotinic Acid	1.0
Pyridoxine-HCl	1.0
Sucrose	30000.0
Thiamine-HCl	0.5

pH 5.6

Procedure

Plant material was cultured as above in the Strawberry regeneration experiments. Micropropagated blackcurrant plants, however, were subcultured at 3 week intervals. All explant material for the experiments was taken from plant material on the 3rd week of

culture. Again all experiments were carried out in a lamina flow hood.

NA described in chapter 3, page 66 was also used in this section. Again the media were prepared as above.

Hormones used.

6-Benzylaminopurine (BAP)
2,4-Dichlorophenoxyacetic acid (2,4-D)
Gibberellic acid (GA)
Indole-3-acetic acid (IAA)
Indole-3-butyric acid (IBA)
Kinetin (K)

General conditions for blackcurrant experiments.

Preparation of plant material.

Leaf discs and internodal segments were excised from axenic, vigorous, young microplants on the third week after culture. The leaf discs and internodal segments were prepared as in the previous regeneration experiments. Again ten explants were placed into each petri dish, leaf discs with their abaxial surface uppermost, and sealed with "Nescofilm".

Internodal segment and leaf disc experiments.

Specific conditions for each experiment.

Experiment 4.2.1.

Genotype: Ben More
Baldwin.

Number of segments: 60 segments / 2 genotypes / 4 treatments.

Choice of media: NA, successful in Rubus regeneration experiments.

Choice of hormones:

1. BAP 2 mg/l and IBA 0.1 mg/l.
2. BAP 0.2 mg/l and 2,4-D 0.2 mg/l.

Both of the above hormone combinations were successful in the regeneration of shoots from Rubus spp.

Experimental conditions: As exp.1 section 4.1. except that half of the explants were grown under conditions of 24 hour darkness.

Comment: Medium NA successful in Rubus and Fragaria spp. was used in this experiment as a starting point in the regeneration of blackcurrants.

Results Experiment 4.2.1.

In this experiment, healthy callus production and shoot regeneration occurred on medium NA containing both of the hormone combinations used (0.1 mg/l IBA with 2

mg/l BAP and 0.2 mg/l BAP with 0.2 mg/l 2,4-D), from blackcurrant internodal segments grown under the light regime. No shoot regeneration occurred in the dark, only white unhealthy, callus was produced.

Table 4.2.1. The effect of 2 different hormone combinations in medium NA on 60 peeled internodal segments of 2 blackcurrant genotypes.

Hormone combin.	Regime	Genotype	callused	No.regn	% regn
1	Light	Ben More	9	37	62
	Light	Baldwin	13	33	55
	Dark	Ben More	27	0	0
	Dark	Baldwin	24	0	0
2	Light	Ben More	32	5	8
	Light	Baldwin	27	9	15
	Dark	Ben More	17	0	0
	Dark	Baldwin	19	0	0

This experiment was examined statistically using a generalised linear model with binomial errors logit link function. This analysis showed that the light regime and hormone combination were very significant ($P < 0.001$ and $P < 0.01$ respectively) in determining the regeneration potential, but the cultivar effect was not significant.

The following figure (4.2) illustrates the regeneration of blackcurrant internodal segments of the cv. Ben More on NA containing 0.1 mg/l IBA and 2 mg/l BAP.

Fig. 4.2



Plantlet formation from internodal segments of the blackcurrant cv. Ben more on medium NA with the hormones IBA and BAP.

Experiment 4.2.2.

Genotype: Ben More

Number of segments: 20 internodal segments / 1
genotype / 1 treatment.

Choice of media: NA

Choice of hormones: IBA at 0.1 mg/l and BAP 2
mg/l

Experimental conditions: As exp.4.1.1

Comment: This experiment examined the number of
plantlets that could be produced from 20
internodal segments over a period of 4, 12 and 20
weeks.

Results Experiment 4.2.2.

As can be seen from table 4.2.2a on the following
page the number of plantlets increases over time.
However, the number of internodal segments regenerating
does not increase significantly.

Table 4.2.2a. Number of plantlets produced over time from 20 segments. No. of segments regenerating are in brackets.

No. segments	4 weeks	12 weeks	20 weeks
20	20 (12)	45 (12)	77 (13)

Table 4.2.2b. Average productivity per explant at 4, 12 and 20 weeks.

Average	4 weeks	12 weeks	20 weeks
productivity	1.0	2.25	3.85

Table 4.2.2c. Number of plantlets produced from 20 individual internodal segments.

Segment number	4 weeks	12 weeks	20 weeks
1	2	2	6
2	0	0	1
3	1	3	4
4	3	5	9
5	0	0	0
6	1	6	6
7	1	3	3
8	0	0	0
9	1	1	5
10	1	4	5
11	2	7	12
12	0	0	0
13	0	0	0
14	0	0	0
15	1	1	4
16	2	2	5
17	0	0	0
18	0	0	0
19	3	6	9
20	2	5	9

The above table (4.2.2c) illustrates the general variability in regeneration between individual internodal segments.

Experiment 4.2.3.

Genotype: Ben More
Baldwin.

Number of discs: 40 discs / 2 genotypes / 2
treatments.

Choice of media: NA.

Choice of hormones:

1. BAP 2 mg/l and IBA 0.1 mg/l.
2. BAP 0.2 mg/l and 2,4-D 0.2 mg/l.

Experimental conditions: As exp.4.1.1

Comment: NA with the two hormone combinations used
in experiment 4.2.1 was used here to determine
any effects with leaf tissue.

Results Experiments 4.2.3.

In this experiment no plantlet regeneration was
achieved from the discs and very little callus was
produced.

Table 4.2.3. The effect of media NA and 2 different hormone combinations (BAP 2 mg/l with IBA 0.1 mg/l and BAP 0.2 mg/l with 2,4-D 0.2 mg/l) on 2 blackcurrants.

Hormone comb.	Genotype	No. discs	No. died	No. callused
1	Ben More	20	20	0
	Baldwin	20	19	1
2	Ben More	20	17	3
	Baldwin	20	19	1

Due to the very poor result with NA medium (previously successful in Rubus and Fragaria spp.), the next experiment examined the effects of an increased level of sucrose in NA medium with the same hormone combinations used in exp.4.2.3.

Experiment 4.2.4.

Genotype: Ben More
Baldwin.

Number of discs: 40 discs / 2 genotypes / 2 treatments.

Choice of media: NA with 30 g/l sucrose.

Choice of hormones:

1. BAP 2 mg/l and IBA 0.1 mg/l.
2. BAP 0.2 mg/l and 2,4-D 0.2 mg/l.

Experimental conditions: As exp.4.1.1

Comment: NA with the two hormone combinations used in experiment 4.2.3, was used here with an increased sucrose concentration to determine any effects on leaf tissue.

Results Experiments 4.2.4.

In this experiment again no plantlet regeneration was achieved from the discs, and very little callus was produced.

Table 4.2.4. The effect of media NA with 30 g/l sucrose and 2 different hormone combinations (BAP 2 mg/l with IBA 0.1 mg/l and BAP 0.2 mg/l with 2,4-D 0.2 mg/l) on Ben More and Baldwin.

Hormone comb.	Genotype	No. discs	No. died	No. callused
1	Ben More	20	20	0
	Baldwin	20	20	0
2	Ben More	20	17	3
	Baldwin	20	20	0

Poor results were still obtained from NA with an increased sucrose concentration and BM was used in the next experiment in an attempt to support cell proliferation.

Experiment 4.2.5.

Genotype: Ben More

Baldwin.

Number of discs: 20 discs / 2 genotypes / 3 treatments.

Choice of media: BM with 20 g/l and 30 g/l sucrose.

Choice of hormones:

1. BAP 2 mg/l and IBA 0.1 mg/l.
2. BAP 0.2 mg/l and 2,4-D 0.2 mg/l.
3. BAP 0.5 mg/l and IBA 2 mg/l.
4. 2,4-D 0.2 mg/l.

Experimental conditions: As exp.4.1.1

Comment: BM with four different hormone combinations and two sucrose concentrations was examined for ability to induce plantlet regeneration.

Results Experiment 4.2.5.

As shown in table 4.2.5 on the following page, no plantlet regeneration occurred from BM under any of the hormone combinations or sucrose concentrations, and very little callus was produced. Alternative hormone combinations were therefore used in the next two

experiments in an attempt to support cell proliferation or induce plantlet regeneration.

Table 4.2.5. The effect of BM with 4 different hormone combinations and 2 different sucrose concentrations on Ben More and Baldwin.

Hormone comb.	Genotype	No. discs	No. died	No. callused
30 g/l sucrose				
1	Ben More	20	19	1
	Baldwin	20	20	0
2	Ben More	20	18	2
	Baldwin	20	19	1
3	Ben More	20	20	0
	Baldwin	20	20	0
4	Ben More	20	18	2
	Baldwin	20	19	1
20 g/l sucrose				
1	Ben More	20	19	0
	Baldwin	20	20	0
2	Ben More	20	17	3
	Baldwin	20	19	1
3	Ben More	20	19	1
	Baldwin	20	20	0
4	Ben More	20	18	2
	Baldwin	20	18	2

Alternative hormone combinations were used in the next two experiments in an attempt to support cell proliferation or induce plantlet regeneration.

Experiment 4.2.6.

Genotype: Ben More

Baldwin.

Number of discs: 20 discs / 2 genotypes / 4 treatments.

Choice of media: BM

Choice of hormones:

1. Kinetin 2 mg/l and IAA 0.1 mg/l.
2. Kinetin 2 mg/l and 2,4-D 0.2 mg/l.
3. Kinetin 2 mg/l and 2,4-D 0.1 mg/l.

Experimental conditions: As exp.4.1.1

Comment: Alternative hormone combinations involving the cytokinin kinetin were incorporated into BM and used in this experiment.

Results Experiment 4.2.6.

Again as in the previous leaf disc experiments, the following table (table 4.2.6) shows that no regeneration occurred from any of the leaf discs and very little callus was produced. The next experiment examined a further two hormone combinations and two sucrose combinations in BM.

Table 4.2.6. Effect of BM and 3 hormone combinations on Ben More and Baldwin.

Hormone comb.	Genotype	No. discs	No. died	No. callused
1	Ben More	20	20	0
	Baldwin	20	20	0
2	Ben More	20	18	2
	Baldwin	20	20	0
3	Ben More	20	20	0
	Baldwin	20	19	1

Experiment 4.2.7.

Genotype: Jet

Number of discs: 30 discs / genotype / 4 treatments.

Choice of media:

1. BM with 20 g/l sucrose.
2. BM with 30 g/l sucrose.

BM, with 2 different sucrose combinations, and 2 hormone combinations (including Gibberellic acid not previously used in the blackcurrant regeneration experiments) was used here.

Choice of hormones:

1. IBA 0.1 mg/l, BAP 2 mg/l and GA 2.5 mg/l.
2. GA 2.5 mg/l.

Experimental conditions: As exp.4.1.1

Comment: Effect of two different sucrose concentrations in BM was examined here.

Results Experiments 4.2.7.

As can be seen from the results in the table below, very little callus and no regeneration was produced from any of the 4 treatments used. The little callus that was produced on the few discs died soon after formation.

Table 4.2.7. Effect of BM with 2 levels of sucrose and 2 different hormone combinations on the blackcurrant cultivar Jet.

Hormone comb.	Sucrose	No. discs	No. callus	No. died
IBA, BAP, GA	20 g	30	2	28
IBA, BAP, GA	30 g	30	3	27
GA	20 g	30	2	28
GA	30 g	30	1	29

Due to the poor results obtained for leaf disc survival under all of the media and hormone conditions used, it was feared that some compounds given out by the leaf disc, due to the wounding, were killing the tissue. Activated charcoal was added in an attempt to absorb any toxins present, thus allowing cell growth to occur.

Experiment 4.2.8.

Genotype: Jet

Number of discs: 30 discs / 1 genotype / 8 treatments.

Choice of media:

1. NA with 20 g/l activated charcoal and 20 g/l sucrose.
2. NA with 20 g/l activated charcoal and 30 g/l sucrose.
3. NA without charcoal and 20 g/l sucrose.
4. NA without charcoal and 30 g/l sucrose.

Choice of hormones:

1. IBA 0.1 mg/l, BAP 2 mg/l and GA 2.5 mg/l
2. GA 2.5 mg/l.

Experimental conditions: As exp.4.1.1

Comment: NA, with and without activated charcoal and with 2 different sucrose concentrations, was used in this experiment along with 2 different hormone combinations in an attempt to support growth of blackcurrant leaf tissue.

Results Experiment 4.2.8.

Explant survival was very poor on all 8 treatments as illustrated in table 4.2.8.

Table 4.2.8. Effect of NA with 2 different sucrose concentrations, each with and without activated charcoal and with 2 different hormone combinations on 30 discs of Jet.

Hormone comb.	Sucrose	Charcoal	Callus	Died
IBA, BAP, GA.	20 g	+	0	30
IBA, BAP, GA.	30 g	+	0	30
IBA, BAP, GA.	20 g	-	3	27
IBA, BAP, GA.	30 g	-	6	24
GA.	20 g	+	0	30
GA.	30 g	+	0	30
GA.	20 g	-	3	27
GA.	30 g	-	0	30

Activated charcoal containing medium appeared to almost completely inhibit callus production, and a reduced salts medium with various sucrose and hormone combinations were used in the next experiment.

Experiment 4.2.9.

Genotype: Baldwin

Number of discs: 40 discs / 2 genotypes / 6 treatments.

Choice of media: 1/2 M+S, 30 g sucrose, 7 g agar
1/2 M+S, 20 g sucrose, 7 g agar

- Choice of hormones: 1. IBA 0.1 mg/l and BAP 2
mg/l
2. GA 2.5 mg/l
3. BAP 0.2 mg/l and 2,4-D 0.2
mg/l

Experimental conditions: As exp.4.1.1

Comment: A reduced salts medium was used in this experiment in an attempt to achieve explant survival on the media.

Results Experiment 4.2.9.

A reduced salts medium appeared to have little effect on the ability of the explants to survive and grow.

Table 4.2.9. Effect of a reduced salts medium on explant survival.

	No. discs	Died	Callused
20 g sucrose			
Hormone comb.1	40	35	5
Hormone comb.2	40	40	0
Hormone comb.3	40	40	9
30 g sucrose			
Hormone comb.1	40	37	3
Hormone comb.2	40	40	0
Hormone comb.3	40	40	4

Due to the complete lack of success obtained for leaf disc survival under any of the treatments, I decided to

examine the physical conditions, rather than continue examining other media and hormone combinations.

Experiment 4.2.10.

Genotype: Baldwin

Number of discs: 70 discs / 1 genotype / 4 treatments.

Choice of media: 1/2 M+S, 20 g sucrose 7 g agar.

Choice of hormones: No hormones

IBA 0.1 mg/l and BAP 2 mg/l

Experimental conditions: Half of the discs were treated as exp.4.1.1 under the light banks and the other half were left on the laboratory bench.

Comment: As no successful leaf disc survival had been achieved under any of the treatments used this far, it was decided to remove the discs from the light banks and leave them on the laboratory bench with reduced lighting.

Results experiment 4.2.10.

As can be seen from the results in table 4.2.10, significant differences occurred between explant

survival under the light banks and on the laboratory bench. Callus production was achieved from explants placed onto hormone containing medium.

Table 4.2.10. The effect of lighting conditions on explant survival.

	No explants	Hormones	Died	No. Callus	% Callus
Light	70	Present	70	0	0
Banks	70	Absent	70	0	0
Lab	70	Present	14	43	61
Bench	70	Absent	11	10	14

As is evident from table 4.2.10, successful callus production was achieved from the discs on the laboratory bench away from the fluorescent light banks, in hormone containing medium. Experiments are now proceeding to induce regeneration from the leaf discs, by placing onto the medium described in this experiment containing various hormone combinations and other alternatives.

Section 4.3. The regeneration of Vaccinium spp.

Materials and Methods.

Genotypes Used.

cv. North Sky

cv. North Country

Maintenance of Plant Genotypes.

Plant genotypes were maintained in vitro on the following medium.

Woody Plant Medium (WPM)

	mg/l
NH ₄ NO ₃	400
Ca(NO ₃) ₂ ·4H ₂ O	556
K ₂ SO ₄	990
MgSO ₄ ·7H ₂ O	370
KH ₂ PO ₄	170
CaCl ₂ ·H ₂ O	96
MnSO ₄ ·H ₂ O	16.9
ZnSO ₄ ·7H ₂ O	8.6
CuSO ₄ ·5H ₂ O	0.25
H ₃ BO ₃	6.2
NaMoO ₄ ·2H ₂ O	0.25
FeSO ₄ ·7H ₂ O	27.8
Na ₂ EDTA	37.
Agar	7000.0
DMAAP	5.0
Glycine	2.0
Inositol	100.0
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Sucrose	20000.0
Thiamine-HCl	1.0

pH 5.3

Ref: LLoyd and McCown 1980.

Constituents of media for blueberry regeneration experiments not already described in previous chapters.

1/2 M+S medium	
NH ₄ NO ₃	825.0
KNO ₃	950.0
CaCl ₂ ·2H ₂ O	220.0
MgSO ₄ ·7H ₂ O	185.0
KH ₂ PO ₄	85.0
FeNa.EDTA	18.35
H ₃ BO ₃	3.1
MnSO ₄ ·7H ₂ O	11.15
ZnSO ₄ ·7H ₂ O	4.3
KI	0.415
Na ₂ MoO ₄ ·2H ₂ O	0.125
CuSO ₄ ·5H ₂ O	0.0125
Glycine	1.0
Inositol	50.0
Nicotinic acid	0.25
Pyridoxine-HCl	0.25
Thiamine-HCl	0.05

Other media used

NA, A and WPM described in chapter 3, page 66.

The sucrose and agar concentrations were maintained at a constant level in all the above media (20 g/l and 7 g/l respectively).

Hormones Used

6-Benzylaminopurine (BAP)
 6- α -Dimethylallylaminopurine (DMAAP)
 Indole-3-butyric acid (IBA)

Procedure

As before, all explant material was obtained from axenic, vigorous young microplants, this time on the third week after subculture. With blueberries, the

leaves were too small to cut discs from, and explants were therefore prepared by slicing the leaf in half along the mid-vein (unless otherwise stated). The discs were placed with the abaxial surface uppermost on the various experimental media.

General conditions for leaf tissue experiments.

Preparation of leaf material.

Leaf explants from blueberries were prepared as described above, and the leaf pieces placed abaxial surface uppermost on the media.

Arrangement of the discs.

The discs were arranged as for the strawberry regeneration experiments.

Leaf section experiments.

Specific conditions for each experiment.

Experiment 4.3.1.

Genotype: North Sky.

North Country.

Number of leaf sections: 50 sections / 2 genotypes
/ 4 treatments.

Choice of media: NA

A

1/2 M+S

WPM

Four different media were used in this experiment in an attempt to achieve shoot regeneration from blueberry leaf sections. Media A and NA had previously been successful in Rubus spp., WPM as it is routinely used in the micropropagation of woody plants, and 1/2 M+S was used to reduce the ionic concentration of M+S salts (as WPM has a low ionic concentration).

Choice of hormones: IBA 0.1 mg/l and BAP 2 mg/l.

Experimental conditions: As exp.1 section 4.1.

Comment: Four media were examined in this experiment for their ability (if any) to induce plantlet regeneration from blueberry leaf tissue.

Results Experiment 4.3.1

Two of the four media used in this experiment gave a small amount (between 6 and 20%) of plantlet regeneration. Callus production was successfully achieved on all 4 media; however, the callus produced on

1/2 M+S and WPM was greener and healthier than that produced on the other 2.

Table 4.3.1. The effect of media NA, A, 1/2 M+S and WPM containing the hormone combination IBA at 0.1 mg/l and BAP at 2 mg/l, on 50 leaf sections each of 2 blueberry genotypes.

Medium	Genotype	No. callus	No. Regn.	% Regn.
NA	North Sky	14	3	6
	North Country	17	5	10
A	North Sky	21	0	0
	North Country	17	0	0
1/2 M+S	North Sky	27	10	20
	North Country	32	9	18
WPM	North Sky	22	0	0
	North Country	17	0	0

Experiment 4.3.2.

Genotype: North Sky.

North Country.

Number of leaf sections: 50 sections / 2
genotypes / 4 treatments.

Choice of media:

NA.

A.

1/2 M+S.

WPM.

Choice of hormones: BAP 0.2 mg/l and 2,4-D 0.2 mg/l.

Experimental conditions: As exp.4.1.1

Comment: The effect of BAP and 2,4-D was examined on plantlet regeneration.

Results Experiment 4.3.2.

This experiment examined the effect of a different hormone combination (in the same media as exp.4.3.1) on the ability of leaf tissue to regenerate. This time, however, no regeneration was achieved, illustrating the importance of the correct hormone conditions for regeneration.

Table 4.3.2. The effect of media NA, A, 1/2 M+S, and WPM containing the hormone combination BAP 0.2 mg/l and 2,4-D on 50 explants each of 2 blueberry genotypes.

North Sky Blue			
Medium	Genotype	No. callus	No. regn.
NA	North Sky	34	0
	North Country	41	0
A	North Sky	34	0
	North Country	32	0
1/2 M+S	North Sky	37	0
	North Country	39	0
WPM	North Sky	36	0
	North Country	27	0

Experiment 4.3.3.

Genotype: North Sky.

North Country.

Number of leaf sections: 50 sections / 2 genotype
/ 4 treatments.

Choice of media: NA and WPM.

Choice of hormones: 1. DMAAP at 2 mg/l
2. DMAAP at 3 mg/l

Experimental conditions: As exp.4.1.1

Comment: This experiment examined the effect on
plantlet regeneration of a hormone commonly used in
blueberry micropropagation medium, DMAAP.

Results Experiment 4.3.3.

Here one concentration (3 mg/l) of the hormone DMAAP,
proved successful at regeneration in both media
examined, but the other concentration (2 mg/l) only
induced callus production.

Table 4.3.3. The effect of 2 concentrations of the hormone Dimethylallylamino-purine on 50 leaf sections each of 2 blueberry genotypes in media WPM and NA.

Medium	Hormone mg/l	Genotype	No. callus	No. regn	% regn
WPM	2	NS	27	0	0
WPM	2	NC	32	0	0
NA	2	NS	15	0	0
NA	2	NC	32	0	0
WPM	3	NS	17	24	48
WPM	3	NC	26	18	36
NA	3	NS	12	11	22
NA	3	NC	15	9	18

The figure (4.3) on the following page shows plantlet regeneration from leaf sections of North Country on WPM with 3 mg/l DMAAP. A large number of plantlets can be seen forming on each leaf section.

Fig. 4.3



Plantlet regeneration from leaf sections of the blueberry cv. North Country on WPM containing 3 mg/l DMAAP.

Experiment 4.3.4.

Genotype: North Sky.

Number of leaf sections: 50 sections / 1 genotype
/ 3 treatments.

Choice of media: WPM.

Choice of hormones: DMAAP at 3 mg/l

Experimental conditions: As exp.4.1.1 except that
leaf sections were produced in 3 ways as follows:

- 1) Cutting in half along the mid-vein.
- 2) Cutting the edges off the leaf and leaving the
mid-vein intact.
- 3) Whole leaf

Comment: This experiment examined the effect on
regeneration of leaf sections produced in different
ways.

Results Experiment 4.3.4.

Table 4.3.4. Effect of the type of leaf section on
plantlet regeneration.

Type of section	No. Callus	No. Regn.	% Regn.
1	19	22	44
2	22	19	38
3	9	2	4

Significant differences ($P < 0.001$) occurred between the ability of different types of leaf section to regenerate. The low amount of regeneration from whole leaf tissue was probably due to a lack of wounding on the leaf. Wounding appears to encourage cell proliferation leading to regeneration.

Experiment 4.3.5.

Genotype: North Sky.

North Country.

Number of leaf sections: 50 sections / 2 genotypes
/ 3 treatments.

Choice of media: NA.

Choice of hormones:

1. IBA 0.1 mg/l and DMAAP 2 mg/l.
2. IBA 0.1 mg/l, BAP 2 mg/l and 2,4-D
0.2 mg/l.

Experimental conditions: As exp.4.1.1

Comment: In this experiment the effect of 2 different hormone combinations were examined. IBA and DMAAP were examined together to determine

if IBA, with DMAAP, would increase the amount of regeneration. IBA, BAP and 2,4-D were placed together again to examine if regeneration could be increased.

Results Experiment 4.3.5.

A small amount of regeneration occurred on NA medium containing the IBA, DMAAP hormone combination.

Table 4.3.5. The effect of the hormone combinations 1. IBA and DMAAP and 2. IBA, BAP and 2,4-D in medium NA on 2 blueberry cultivars.

Hormone comb.	Genotype	No. callus	No. regn.	% regn.
1	NS	14/40	2/40	5
	NC	19/40	3/40	8
2	NS	39/40	0/40	0
	NC	32/40	0/40	0

The addition of IBA to NA medium containing DMAAP appeared to reduce the ability of explants to regenerate (compare with exp. 4.3.3, where overall, 20% regeneration occurred). IBA, BAP and 2,4-D very successfully induced callus production but not plantlet regeneration.

Experiment 4.3.6.

Genotype: North Country

Number of discs: 40 discs / 1 genotype / 1 treatment.

Choice of media: WPM.

Choice of hormones: DMAAP 3 mg/l.

Experimental conditions: As exp.4.1.1 except the explants were maintained under conditions of 24 hour darkness.

Comment: The effect of constant darkness on explant regeneration was examined here. Control explants were examined under the lighting conditions described.

Results Experiment 4.3.6.

Table 4.3.6. The effect of 24 hour dark conditions on shoot regeneration from the cv. North Country.

Conditions	No. discs	No. callused	No. regn.	% regn.
Light	40	17	21	53
Dark	40	30	10	25

In this experiment, unlike the Rubus, Ribes or Fragaria experiments, regeneration occurred from leaf material in the dark.

Although regeneration did occur in the dark the number of explants regenerating was reduced from 53% under the light conditions described.

The white plantlets that were produced (as shown in figure 4.4 on the following page) however, failed to produce normal leaves when transferred into the 16 hour light:8 hour dark regime previously described.

Fig. 4.4



The regeneration of white plantlets on leaf sections of the blueberry North Country under conditions of 24 hour darkness.

Experiment 4.3.7.

Genotype: North Country

Number of discs: 20 discs / 1 genotype / 1 treatment.

Choice of media: WPM.

Choice of hormones: DMAAP 3 mg/l.

Experimental conditions: As exp.4.1.1

Comment: The productivity of leaf disc regeneration, rather than the number of discs regenerating, was examined in this experiment.

Results Experiment 4.3.7.

The results shown in table 4.3.7a illustrate that again, as in the strawberry productivity study, the number of plantlets increases over time, however the number of discs regenerating does not increase over time to any significant level.

Table 4.3.7a. Number of plantlets produced over time from 20 leaf discs. No. of discs regenerating are in brackets.

No. discs	4 weeks	12 weeks	20 weeks
20	30 (10)	58 (10)	91 (13)

Table 4.3.7b. Average productivity per explant (from 20 explants) at 4, 12 and 20 weeks.

Average Productivity	4 weeks	12 weeks	20 weeks
	1.5	2.9	4.55

Table 4.3.7c. Number of plantlets produced from 20 individual leaf discs.

Disc number	4 weeks	12 weeks	20 weeks
1	6	6	17
2	0	0	2
3	3	7	7
4	4	9	10
5	0	0	0
6	2	4	6
7	2	6	6
8	1	2	4
9	0	0	0
10	2	2	2
11	0	0	0
12	0	0	0
13	4	9	9
14	5	9	17
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	1
19	0	0	3
20	1	6	7

Experiment 4.3.8.

Genotype: North Country.

No. of discs: 20 discs / 1 genotype / 1 treatment, repeated 5 times.

Choice of media: WPM.

Choice of hormones: DMAAP 3 mg/l.

Experimental conditions: As exp.4.1.1

Comment: The variability in regeneration experiments was examined in this experiment.

Results Experiment 4.3.8.

This experiment very clearly illustrates the variability obtained in the regeneration experiments.

Table 4.3.8. Shoot regeneration from 5 replicates of the same experiment.

Replicate no.	No. discs	No. regen.	% regen.
1	20	9	45
2	20	20	100
3	20	6	30
4	20	0	0
5	20	10	50

The range of leaf disc regeneration results obtained in this experiment varied widely, from one replicate, where none of the discs regenerated, to in another replicate, where all of the discs regenerated. A Chi-squared goodness of fit test was used to determine if the differences which occurred were significant. The results of this test showed that the differences in regeneration which occurred between different replicates, under the same treatment, were highly significant ($P < 0.001$).

Discussion.

In this chapter details are provided of the steps involved in the development of regeneration systems for Fragaria, Ribes and Vaccinium species.

Initially, the regeneration conditions adopted were the medium composition, hormone combinations, leaf disc orientation and the environment used for Rubus spp.

Shoot regeneration was achieved from leaf discs of Fragaria spp. on medium NA containing the hormone combination BAP (0.2 mg/l) and 2,4-D (0.2 mg/l). The regenerative ability of explants varied between genotypes, as illustrated in experiment 4.1.3, where 35% of discs from selection 71WC64 and 17% of discs from selection E2a regenerated. Variability also occurred within the same genotype, under the same conditions in different experiments, as demonstrated by plantlet regeneration from leaf discs of the cultivar Rapella which varied between 20% and 50%.

The strawberry regeneration experiments demonstrate the importance on explant regeneration of medium composition. Three different media (NA, Jones and SMM) with the same hormone combination (0.2 mg/l BAP and 0.2 mg/l 2,4-D), were examined for ability to induce plantlet regeneration. Only one of the three media (NA) induced shoot regeneration, whereas another (Jones) induced root regeneration. The third medium (SMM) was unable to support explant survival and no cell growth

occurred. Only slight differences occurred between NA and Jones media, NA containing nicotinic acid, pyridoxine-HCL and glycine all of which were absent from Jones medium.

Jones medium, although unable to induce shoot regeneration from the three genotypes used in my experiments, had previously been found by Jones et al. (1988) to be successful at regenerating 6 out of the 9 genotypes they tested (including Rapella used in my experiments) from callus produced. This medium induces callus formation from which shoots are subsequently produced, and is not ideal for transformation due to the increased risk of somaclonal variation. I did however find root regeneration from 2 of the 3 genotypes examined at a level of 15%, but these roots could not be induced to form shoots. This experiment illustrated the general variability found throughout regeneration experiments which could be due to the condition of the plant material or to the micropropagation conditions of the stock tissue cultures.

A second medium, developed by Liu and Stanford (1988), (again not ideal for use in a transformation system for the same reason as Jones medium) which proved successful at shoot regeneration from explants (79%) of 1 of 2 genotypes tested, proved successful in my experiments, on 2 of the 3 cultivars tested, but to a much reduced level (15% shoot regeneration). This medium differed from NA in the hormone composition, since it

also lacked nicotinic acid, pyridoxine-HCL and glycine and contained casein hydrolysate.

Because hydrolysate-containing medium supported regeneration, it was decided to examine its effect in SMM, a medium which had proved unsuitable for growth (exp.4.1.1). Casein hydrolysate had a very marked effect on the ability of SMM to support cell growth and division. This component may have been beneficial by providing a source of nitrogen or it may have overcome some deficiency in the medium. These explanations seem unlikely, however, as the simple medium NA could support growth and regeneration. Murashige and Skoog (1962) found that the addition of casein hydrolysate to a medium induced organ formation over a broader range of hormone concentrations than that which normally occurred. This component was therefore incorporated again into SMM with the hormone combination 0.2 mg/l BAP and 0.2 mg/l 2,4-D which was previously shown to have had limited success in shoot regeneration (20%) (exp.4.1.2), in an attempt to improve shoot regeneration. However only callus production was achieved under these conditions.

Experiment 4.1.10 examined the ability of strawberry base tissue to regenerate whole plants. In this experiment the importance of combining the correct medium and hormone combination was clearly shown; when two media and two hormone combinations were examined, each medium induced plantlet regeneration successfully

with only one hormone combination (SMM with IBA and BAP, and NA with BAP and 2,4-D).

In Ribes spp. an internodal segment regeneration system was developed. Although this was relatively effective depending on the hormone combination incorporated into NA medium (60% with IBA and BAP), the development of a leaf disc system is still desirable, as the production of internodal segments from blackcurrants in tissue culture is difficult and time consuming due to their short length in vitro.

The regeneration of shoots from blackcurrant leaf tissue has not so far been achieved. The leaf material fared very poorly on NA, 1/2 M+S and BM media at all the hormone combinations and the sucrose concentrations used under the lighting conditions described. It was feared that phenolic compounds, or other products, diffusing into the medium from the wounded leaf tissue were inhibiting regeneration and activated charcoal was added to absorb them. This substance incorporated into the medium, however, appeared to completely inhibit callus production and could not support explant survival.

In Ribes experiment 4.2.10, significant progress was made towards the development of leaf tissue regeneration conditions, when explant material was left to grow on the laboratory bench rather than put under the light banks. Here, callus production was successfully achieved on 61% of the leaf discs examined. Experiments are now continuing on leaf disc regeneration, which due to the results of exp.4.2.10 looks feasible.

Regeneration of shoots from Vaccinium spp. was achieved on a wider range of media (NA, 1/2 M+S medium and WPM) than the other species examined. With medium NA, regeneration was achieved when two different hormone conditions (IBA 0.1 mg/l and BAP 2 mg/l at a level of 10% and with 3 mg/l DMAAP at 22%) were incorporated. With WPM, regeneration was only achieved with the hormone DMAAP at a concentration of 3 mg/l on around 50% of leaf sections. The other hormone combinations incorporated into WPM (IBA and BAP, BAP and 2,4-D and DMAAP at 2 mg/l) failed to induce plantlet formation. With 1/2 M+S medium regeneration was achieved with the hormone combination IBA 0.1 mg/l and BAP 2 mg/l at a level of around 20%. The effects of the hormone DMAAP in this medium have yet to be examined.

In the productivity experiments, similar results were obtained from all 3 species. These experiments demonstrated the increase in shoot production over time but also showed that generally the number of discs regenerating did not increase over time.

The variability in regeneration experiments was widest in Vaccinium spp., with one replicate yielding 0% regeneration, and another yielding 100% regeneration. In Ribes spp., the variation between internodal segment regeneration was very small in the different experiments and was therefore not examined further.

The regeneration techniques developed above and in chapter 3 for Rubus spp. varied in the levels of success

at which shoot regeneration could be accomplished.

However as some degree of regeneration was produced for all four species., experiments on Agrobacterium transformation could be carried out.

The next chapter deals with the use of marker genes in the development of transformation systems and the preliminary experiments necessary for their use in Rubus, Ribes, Vaccinium and Fragaria spp.

Chapter 5.

**The use of marker genes in the study of soft fruit
transformation.**

The use of marker genes in the study of soft fruit transformation.

Introduction.

The need for marker genes, to show the occurrence of transformation, became necessary following the development and use of disarmed isolates of Agrobacterium.

As described in chapter 2, when Agrobacterium infects plant material a disease known as crown gall occurs. This condition is undesirable, causing difficulties in the regeneration of whole plants from explant material. This effect on regeneration is due to the high levels of phytohormones in transformed tissues, produced by expression of genes on the T-DNA. It is therefore undesirable to inoculate plant material with wild type isolates which cause crown gall or hairy root disease.

Disarmed isolates were produced (Ooms et al. 1982b) to overcome this problem, by either partial or total deletion of the T-DNA in the Ti-plasmid.

Prior to the deletion of genes on the T-DNA, both the presence of opines, and the ability of transformed cells to proliferate without added hormones were traits used as an indication of transformation of the plant material. Once one or both of these features were removed, other methods of identification had to be developed.

As described in chapter 1, the large size of the Ti-plasmid makes the insertion of marker genes between the T-DNA borders difficult. Disarming the isolate to remove the disease symptoms, however, does not remove the essential vir functions which encode transfer of the genes in trans from a separate plasmid containing the T-DNA borders. A second plasmid is therefore used which contains the T-DNA borders with a gene between them whose transfer into the plant can be easily detected. Such a gene is known as a marker gene.

There are a number of features which marker genes must have in order to be useful. A selectable marker gene is a gene that, when introduced into plants, is selected as it confers resistance to some substance normally inhibitory to the plant. The substance used must inhibit plant cells not transformed with the gene. For example, if a gene conferring resistance to an antibiotic is inserted into the plant material, non-transformed cells will be killed, or their growth retarded, by the antibiotic so that transformed cells or plants can be selected on the basis of their growth being unaffected by high levels of the antibiotic.

Scorable marker genes, unlike selectable markers, do not confer resistance to an inhibitory substance. As they cannot be used to prevent inhibition of the growth of transformed plants, the relative growth of transformed plants and non-transformed plants cannot be used as an indication of transformation. However, scorable marker genes confer differences on the plant

material which should be easily assayed for, the gene product being a substance which does not normally occur in the plant material.

A number of marker genes are currently in use, and most have advantages and disadvantages depending on the species of plant material being used. Among those genes available are those encoding for the enzymes beta-galactosidase, chloramphenicol acetyl transferase (cat), neomycin phosphotransferase (NPT II), beta-glucuronidase (GUS) and the firefly luciferase.

As well as those genes whose expression leads to disease symptoms, occurring on the T-DNA, other genes encoding unusual amino acids known as opines are present. These opines provide "natural" marker genes (the octopine and nopaline genes) with useful features for the identification of transformants. The octopine synthase (De Greve et al. 1982b) and the nopaline synthase (Depicker et al. 1982 and Bevan et al. 1983a) enzymes do not have any problems of endogenous activity associated with them, as they do not occur in non-transformed tissue, and therefore no false positive results can be obtained. However, the assays are not commonly used as they are time-consuming and laborious to carry out. As well as the disadvantage of the assay method, the octopine synthase cannot tolerate amino terminal fusions, which introduce amino acid substitutions making the enzyme unstable (Jones et al. 1985). With disarmed plasmids where the entire T-DNA has been removed, the genes encoding the above amino acids

are no longer present and other markers have to be inserted.

One of the most widely used selectable marker genes is that encoding the neomycin phosphotransferase type II (NPT II) enzyme, originally isolated from the prokaryotic transposon Tn5 (Becke et al. 1982). The enzyme detoxifies aminoglycoside compounds such as kanamycin, neomycin and G-418 by phosphorylation (rev. Fraley et al. 1986). It is a stable enzyme tolerating amino-terminal fusions and remaining enzymatically active.

In a number of plant species, the antibiotic kanamycin causes visible whitening and/or suppression of growth of non transformed plants eg. spring wheat and barley (Lee et al. 1989), Petunia, carrot, sunflower and tobacco (Fraley et al. 1983), leading to an initial, simple selection procedure for transformed plants.

The NPT II gene, fused to plant transcriptional signals, has been incorporated into numerous plant transformation vectors (Fraley et al. 1983, Bevan 1984, An et al. 1985, Fraley et al. 1985, Van den Elzen et al. 1985, Velten and Schell 1985, Matzke and Matzke 1986, Koncz and Schell 1986, Horsch and Klee 1986).

In addition to visible selection of plants containing the NPT II gene, other methods of assay have been carried out. The first NPT II assays of transformed plant material were done by gel filtration of tissue extracts (to separate the proteins), the collected fractions being assayed for the ability to phosphorylate

kanamycin. This was followed by a second separation system involving paper chromatography to confirm that the radioactive spots were only due to phosphorylated kanamycin (Herrera-Estrella et al. 1983a). In 1984, Reiss et al. developed a sensitive, quantitative method for the detection of NPT II transformed plants. This method involves electrophoretic separation of the enzyme, then antibiotic phosphorylation in an overlaid agarose gel, followed by blotting onto phosphocellulose paper. However this method is not suitable for the screening of a large number of plants and quicker methods were required.

McDonnell et al. (1987) developed a relatively simple assay procedure based on the dot blot assay of Platt and Yang (1987). The assay of McDonnell et al. makes the detection of NPT II activity in transformed tissues quicker and easier with little exposure to radiation. The assay can be done on prepared extracts in approximately 2-3 hours followed by an overnight exposure of the autoradiogram. With this assay, controls have to be carried out on non-transformed plants for any background activity of the enzyme which may be present in the plant material.

Another widely used gene is that encoding the enzyme chloramphenicol acetyltransferase (cat), which is not normally found in plant tissues. The coding sequence of chloramphenicol acetyltransferase (cat) has been combined with the nopaline synthase (nos) promoter (Herrera-Estrella et al. 1983a,b) and is functionally

expressed in plant cells. Transformed plants can be screened for on medium containing chloramphenicol by examining root growth. The enzyme of bacterial origin, provides a sensitive assay system, based on the identification of acetylated chloromphenicol, as no similar endogenous activity is present in plant cells (Herrera-Estrella et al. 1983a).

Other non-selectable markers have been developed for use in plant transformation systems. The firefly luciferase has been used as a marker in transgenic plants. The gene encodes the firefly luciferase enzyme that catalyses the light-producing, ATP-dependent oxidation of luciferin. Extracts from transformed plants produce a light flash detected in a luminometer, in the presence of the substrates luciferin and ATP (Ow et al. 1986). However, the enzyme is labile and difficult to assay with accuracy (DeLuca and McElroy 1978).

Beta-galactosidase gene fusions in plants (Helmer et al. 1984) have been of no use in the assay of transformation due to high endogenous activity in plants.

The last marker gene I will briefly discuss here is that encoding the beta-glucuronidase enzyme. Beta-glucuronidase (encoded by the uidA locus (Novel and Novel 1973)), is a hydrolase that catalyses the cleavage of a wide variety of beta-glucuronides. The natural substrates are probably beta-glucuronide linkages in mucopolysaccharides and various biological glucuronides such as those of steroid hormones (Stoeber 1961). The

enzyme has an absolute requirement for a *B-D*-glucuronide linkage but is very indiscriminating with respect to the aglycone, leading to the use of a variety of synthetic substrates. Beta-glucuronidase (GUS) is very stable, and will tolerate a wide range of undesirable conditions such as the presence of many detergents and widely varying ionic conditions. It is most active in the presence of thiol reducing agents such as beta-mercaptoethanol or DTT. GUS has no co-factors, nor any ionic requirements, although because it is inhibited by some heavy divalent metal ions (Cu_2^+ and Zn_2^+), ethylene diamine tetraacetic acid (EDTA) is included when assaying for the presence of the enzyme (Jefferson 1987a,b).

The *B*-glucuronidase enzyme has a number of features ideal for use in transformation experiments. Firstly; the enzyme is not normally present in plant tissues, and secondly; expression of the enzyme can easily be assayed for, providing clear and conclusive results. Usefulness of the inserted beta-glucuronidase enzyme, however, depends on the availability of suitable substrates for the enzyme. The substrate must, when acted upon by the enzyme, produce a product readily distinguishable from the substrate. Many substrates of *B*-Glucuronidase are available commercially as spectrophotometric, fluorometric and histochemical substrates. The fluorogenic 4-methylumbelliferyl-*B-D*-glucuronide is now the preferred substrate (Paigen 1979). The substrate 4-methyl umbelliferyl-*B-D*-glucuronide is not fluorescent

until cleaved by the enzyme to release the compound 4-methyl umbelliferone, and the change in fluorescence can easily be detected unaided by eye.

The Beta-glucuronidase enzyme can also be assayed by histochemical means. For histochemical localisation of beta-glucuronidase activity in tissues and cells, the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) gives a blue precipitate at the site of enzyme activity.

The markers NPT II and GUS were used in my experiments on the transformation of Rubus, Ribes, Fragaria and Vaccinium spp.

The use of the genes encoding the NPT II and GUS enzymes as markers of transformation in soft fruit, and the binary vectors in which they are carried will be described below, along with the experiments involving their use. This will be done in 3 sections, (5.0) general information and conditions, (5.1) use of the NPT II enzyme and (5.2) use of the GUS enzyme.

Section 5.0 Materials and Methods

Bacterial isolates, their description and maintenance.

Strains	Characteristics	Ref.
<u>Agrobacterium</u>		
516	Wild Type <u>A. tumefaciens</u> , isolated from <u>Rubus</u> sp.	SCRI
LBA4404	Contains plasmid pAL4404 which is SMr, Rif ^r , entire T-DNA has been removed.	Ooms <u>et al.</u> 1982b
Ar9402	Wild Type <u>A. rhizogenes</u> Rif ^r	J. Manners JII
<u>Escherichia coli</u>		
HB101	Non conjugative strain	Maniatis <u>et al.</u> 1982

The above A. tumefaciens and A. rhizogenes isolates (Fox 1965a,b), were kindly supplied by Dr J. Manners of the John Innes Institute. LBA4404 is a disarmed A. tumefaciens isolate in which the entire T-DNA of the wild type octopine Ti-plasmid, pTi-Ach5, has been removed by deletion mutation (Ooms et al. 1982b). LBA 4404 is resistant to the antibiotics streptomycin and rifamycin. 516 is a wild type gall-producing isolate from Rubus spp. Ar9402 is a wild type isolate with a root-inducing (Ri) plasmid, causing hairy root production from which whole plants have been regenerated. This isolate could prove useful as an alternative to transformation with a Ti-plasmid. Ar9402 is resistant to the antibiotic rifamycin.

E. coli strain HB101 is commonly used for maintenance and large-scale production of plasmids.

Maintenance of the isolates.

A. tumefaciens isolates 516 and LBA4404 were maintained on Luria-Bertani (LB) medium, described in chapter 2. LBA4404 medium containing 50 mg/l of the antibiotic rifamycin. A. rhizogenes isolate Ar9402 was maintained on Yeast Mannitol (YM) medium again described in chapter 2, containing 50 mg/l of rifamycin. E. coli was maintained on LB medium.

The isolates were maintained in petri dishes containing the appropriate medium at 4°C (re-streaking onto fresh medium occurred every 3 weeks) until they were required for inoculation.

Binary vectors.

As described in chapter 1, the transfer system of A. tumefaciens requires several modifications before it can be used as a vector. The genes for crown gall morphology must be deleted from the Ti-plasmid and marker genes must be inserted to follow the occurrence of transformation. However, due to difficulties in the manipulation of the Ti-plasmids, the marker gene(s) is inserted into a binary vector, (containing T-DNA borders, which function in trans along with the Vir region on the Ti-plasmid), the binary vector

subsequently being introduced into Agrobacterium. The binary vectors used in the transformation experiment were Bin 19 (kindly supplied by Dr D.M. Lewis of the JII, Norwich) and PBI121 (kindly supplied by Dr D. James of IHR, East Malling).

Bin 19 is a binary vector of approximately 10 Kb. The detailed construction of this vector was described by Bevan (1984). Briefly, this vector contains the neomycin phosphotransferase gene from Tn5, ligated between a nopaline synthase promoter fragment and the nopaline synthase polyadenylation site (Bevan and Flavell 1983). Onto this fragment, T-DNA borders were ligated and this construction was inserted into a wide host range plasmid, pRK252 (derived from RK2) (Figurski and Helinski 1979), that had been modified by the insertion of a kanamycin resistance gene. In the T-region, a fragment containing a number of restriction enzyme sites was also inserted to allow sequences to be inserted into the T-region.

PBI121, (Jefferson 1987a,b) is basically Bin 19 containing the Beta-Glucuronidase coding sequence with the Cauliflower Mosaic Virus (CaMV) 35S promoter (Odell et al. 1985) and the nopaline synthase (nos) terminator ligated into the *Kpn* I site of the Bin 19 polylinker.

Mobilisation of a binary vector into Agrobacterium.

The binary vectors were supplied in E. coli strain HB101 which has no mobilisation functions, therefore a helper plasmid is required to effect conjugation. This process known as triparental conjugation (Ditta et al. 1980) uses the helper plasmid pRK2013 (Bagdasarian et al. 1981 and Figurski et al. 1979).

The triparental cross involves mixing together Agrobacterium into which the binary vector is to be introduced, along with E. coli containing the binary vector and finally E. coli containing the helper plasmid to mobilise Bin 19 into Agrobacterium. In order to select for Agrobacterium containing the binary vector, the antibiotic resistances of each must be considered. As both LBA4404 and Ar9402 are resistant to rifamycin but not kanamycin, and Bin 19 confers resistance to kanamycin but not rifamycin, then after the cross if Agrobacterium is found to be resistant to both, then the binary vector must have been transferred into Agrobacterium. The plasmid pRK2013 is also resistant to kanamycin, however pRK2013 uses and requires the ColE1 replicon for replication (Riedel et al. 1979), and as the ColE1 replicon has a narrow host range, pRK2013 is unable to become established in Agrobacterium, an organism distantly related to E. coli.

The following procedure describes the mobilisation of Bin 19 into Agrobacterium.

The Triparental Cross Procedure.

Bacterial strains, plasmids, antibiotic resistance and medium required for growth.

Strain	Plasmid	Medium	Antibiotic
LBA4404	pAL4404	LB	Rif
Ar9402	pRi9402	YM	Rif
HB101	Bin 19	LB	Kan
HB101	pRK2013	LB	Kan

Media: Yeast Mannitol Broth (YM) and Agar.

Luria-Bertani Broth (LB) and Agar.

Antibiotics: Kanamycin (Kan) and Rifamycin (Rif).

Experimental procedure: Individual overnight cultures of the above bacteria were established in the appropriate broth containing 50 mg/l of the appropriate antibiotic at 28°C in a shaking water bath.

The next day 50 ul of each bacterium was placed individually, in all combinations of 2 and then all combinations of 3, onto circular 2 cm diameter filter paper discs on top of LB medium in 9 cm diameter petri dishes.

The combinations were as follows:

Individuals:

LBA4404

Ar9402

HB101(Bin19)

HB101(pRK2013)

Combinations of 2:

HB101(Bin19) and LBA4404

HB101(Bin19) and Ar9402

HB101(Bin19) and HB101(pRK2013)

HB101(pRK2013) and LBA4404

HB101(pRK2013) and Ar9402

Combinations of 3:

HB101(Bin19), LBA4404, HB101(pRK2013)

HB101(Bin19), Ar9402, HB101(pRK2013)

The above bacterial combinations were incubated on filter paper discs at 25°C for 24 hours.

After 24 hours, each disc was removed and placed into a separate vial containing 10 mls of LB and shaken to distribute the bacteria into the broth. 100 ul from each vial was then removed and plated onto LB medium, containing 50 mg/l each of the antibiotics rifamycin and kanamycin (rif, kan medium). This was incubated for a further 24 hours and then growth observed. Only

Agrobacterium into which Bin 19 had been transferred should grow on the rif, kan medium.

Triparental Cross Results.

The table below shows the results obtained from the triparental cross. In the table, HB101 carrying either pRK2013 or Bin 19 is referred to by the plasmid it contains.

The results obtained from a triparental cross plated on LB medium containing 50 mg/l of the antibiotics rifamycin and kanamycin.

<u>Bacterial combination</u>	<u>presence/absence of growth</u>
LBA4404	small amount of growth
Ar9402	very little growth
pRK2013	no growth visible
Bin19	no growth visible
LBA4404 Bin19	small amount of growth
Ar9402 Bin19	no growth visible
Bin19 pRK2013	no growth visible
pRK2013 LBA4404	small amount of growth
pRK2013 Ar9402	very little growth
Bin19 LBA4404 pRK2013	large amount of growth
Bin19 Ar9402 pRK2013	large amount of growth

The above table demonstrates the difference in growth of the various combinations of bacteria. Obvious differences occurred between the triparental cross and

the other combinations. Since combinations of 2, and individual bacteria still grew on a medium selective for Agrobacterium containing Bin 19, I decided to repeat the cross and plate out the combinations on YM containing 100 mg/l of the antibiotics rif and kan. The following table demonstrates the results obtained from this cross.

The results obtained from a triparental cross plated on YM containing 100 mg/l of the antibiotics rifamycin and kanamycin.

Bacterial combination	presence/absence of growth
LBA4404	no growth visible
Ar9402	no growth visible
pRK2013	no growth visible
Bin19	no growth visible
Bin19 LBA4404	no growth visible
Bin19 Ar9402	no growth visible
Bin19 pRK2013	no growth visible
pRK2013 LBA4404	no growth visible
pRK2013 Ar9402	no growth visible
Bin19 LBA4404 pRK2013	large amount of growth
Bin19 Ar9402 pRK2013	large amount of growth

As can be seen from the table above the second cross plated on a medium (YM) (less suitable for the growth of E. coli and A. tumefaciens) with a higher antibiotic content only allowed the growth of Agrobacterium containing Bin19.

To mobilise PBI121 the above procedure was carried out (replacing HB101 carrying Bin 19 with HB101 carrying PBI121) and selection on YM with 100 mg/l of the antibiotics rif and kan occurred.

Once the binary vector had been inserted into Agrobacterium, inoculations of explant material could then occur, details of which follow.

Procedures for the inoculation of explants with Agrobacterium and regeneration into whole plants.

Preparation of the inoculum.

For inoculation, a loopful of the isolate was taken. This was inoculated into 10 ml of the appropriate broth containing the appropriate antibiotics, and incubated overnight in a shaking water bath at 28°C. After overnight incubation, the bacterial suspension was centrifuged and resuspended in liquid plant medium (NA without hormones or sucrose) to remove the antibiotics. This was repeated twice.

Preparation of leaf discs and internodal segments.

Leaf discs and internodal segments were prepared as described in chapter 3. All plant material for the following experiments was obtained from stock tissue cultures on week 4 of the culture cycle.

Culture of leaf discs and internodal segments.

Leaf discs and internodal segments were grown on the appropriate regeneration medium contained within 9 cm diameter petri dishes described in chapter 3, and grown under the environmental conditions also described in chapter 3.

The inoculation of leaf discs and internodal segments with Agrobacterium containing a binary vector for transformation.

The inoculation of explant material was carried out in a 9 cm diameter petri dish, into which was added 30 mls of an overnight culture of Agrobacterium, resuspended in liquid plant medium (NA, described in chapter 3).

Inoculation was carried out for 20 mins, after which time the explants were removed and incubated (on filter paper discs moistened with liquid NA, on solid NA) for approximately 12 hours (unless otherwise stated in particular experiments).

Regeneration of inoculated explants.

Plant regeneration media described in chapters 3 and 4 were used to induce whole plant regeneration. The environmental conditions were as described in chapter 3

(fluorescent tubes at $70 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$, 16 hour day:8 hour night and grown at a temperature of 20°C).

Methods available to confirm transformation of plants regenerated after inoculation with Agrobacterium carrying a binary vector.

- 1) Southern Hybridisation
- 2) NPT II Assay
- 3) GUS Assay

1) Southern hybridisation of inoculated plants.

The majority of techniques and solutions used in this section were based on those of Maniatis et al. 1982.

Identification of inserted DNA sequences in transformed plants can be accomplished by the transfer technique described by Southern (1975). Genomic DNA is isolated from putative transformants, and digested with one or more restriction enzymes and the resulting fragments are separated on an agarose gel and transferred onto nitrocellulose. The DNA is denatured and mixed with a single stranded radiolabelled probe, prepared from part or whole of the DNA sequence, which the transformation experiment attempted to insert. This procedure allows analysis of plant DNA, to determine whether the desired gene had been inserted. Hybridisation of the plant DNA and the probe occurs if the sequence was inserted.

Isolation of DNA from small amounts of plant material.

Once inoculated and regenerated, the plants are small and therefore very little material is available for DNA isolation. The following technique allows DNA isolation from small (less than 1 g) amounts of plant material.

Extraction Buffer Per 100 ul

Tris 100 uM pH 8

EDTA 20 uM

SDS 0.1%

Diethyldithiocarbamate 0.5%

Plant material was ground with sand and extraction buffer in an Eppendorf tube. The tube was then filled with phenol-chloroform, vortexed for 10 seconds, centrifuged for 2 minutes and the supernatant (S/N) removed to a fresh tube (phenol-chloroform extracts the DNA). The phenol-chloroform extraction was repeated, and 2 volumes of ethanol and 1/10 volume sodium acetate (3 M) were added to the S/N in a fresh tube (this step precipitates the DNA). Here the tube was shaken gently and centrifuged (a pellet of DNA could be seen at this stage) and the S/N removed. The pellet was subsequently dried in a vacuum, dissolved in 1 ml distilled water and RNase was added to remove RNA from the DNA. The phenol-chloroform extraction and ethanol precipitation was repeated as before. (This procedure was kindly supplied by Dr. D. M. Lewis from the John Innes Institute).

Digestion of DNA.

The pellet of DNA obtained from the above procedure was dried in a vacuum concentrator and dissolved in 89 ul H₂O, 10 ul Buffer no 3 (x10) and 1 ul Eco RI. This Eco RI digest was allowed to proceed overnight in a water bath at 37°C.

After the DNA had been digested it was again extracted in phenol-chloroform, precipitated in ethanol, dried and dissolved in a small volume of water (20 ul). 1 ul of loading buffer was added and the DNA was loaded onto an agarose gel (1.8 g agarose in 200 ml TBE). The gel was allowed to run until the loading buffer was within 2 cm of the bottom of the gel. The gel was then cut at the gel wick interface and just below the forward dye position. The gel was also cut along the edges and a plastic lifter used to remove the gel from the gel tank. The lower right hand corner of the gel was trimmed and the gel slid into the staining solution (electrophoresis buffer containing 0.5 ug/ml ethidium bromide) for 30-45 mins at room temperature. After staining the gel was examined and photographed under UV at 302 nm.

Blotting of DNA from gel onto nitrocellulose.

Denaturation Solution

0.2 M NaOH

0.6 M NaCl

Neutralisation Solution

1 M Tris/HCL pH 7.4

1.5 M NaCl

The gel was transferred into denaturation solution for 20 mins, after which it was washed in fresh water and transferred to neutralisation (renaturation) solution for 20 mins.

While the gel was being processed, the nitrocellulose for blotting was prepared. This was done by measuring the gel size and cutting a piece of nitrocellulose (BA85) (whilst wearing clean gloves and working in a clean area) that was 1 mm larger in all directions than the gel and soaking in water for 20 mins then 20 x SSC for at least 5 mins (a corner of the nitrocellulose was cut to match that on the gel).

While the nitrocellulose was soaking, the support for the transfer was prepared. A glass plate was used. This was wider than the gel and wrapped with Whatman 3 MM paper soaked in 10xSSC and suspended across a baking dish. The soaked wick draped over the glass sheet was dipping into the baking dish which contained 10xSSC.

The gel was slid onto the centre of the wick (under surface uppermost) and the bubbles removed using a gloved wetted hand. Excess agar above the wells was removed and the soaked nitrocellulose was placed on top of the gel and the bubbles removed. The gel was then surrounded but not covered with Saran Wrap or film to

prevent liquid from flowing directly from the reservoir to paper towels placed on top of the gel.

The wet nitrocellulose filter was then placed on top of the gel with the cut corners aligned and one edge of the filter extending over the edge of the line of slots on the gel. Two wet pieces of 3 MM paper, cut to exactly the same size as the gel, were placed directly on top of the nitrocellulose filter. A stack of paper towels were placed on top of the 3 MM paper followed by a heavy sheet of plastic and finally a weight.

This was left for 24 hours during which time the paper towels were replaced as they became wet.

After 24 hours the blot stack was removed and the nitrocellulose placed on a clean surface gel side up. The compressed gel was peeled off and examined for signs of bubbles then discarded.

The filter was air dried and baked between 2, 3 MM sheets in a vacuum oven at 80°C for 2 hours.

The DNA immobilised on the filter was then hybridised to a labelled probe.

Preparation of a labelled probe.

The first step in the preparation of a labelled probe was the isolation of the fragment of DNA to be labelled. As both Bin 19 and PBI121 contain the NPT II gene, this was used as a probe. This gene was maintained in plasmid pJIT134 which was a 3.0 Kb ampicillin resistant plasmid.

The 1.0 Kb NPT II gene had an Eco RI restriction site at the 3 prime end and a *Hind* III site at the 5 prime end which could be used to cut the gene from the plasmid.

Mini-Prep of plasmid DNA.

Solution 1

50 mM glucose

10 mM EDTA

25 mM Tris-HCL pH 8

4 ug/ml lysozyme (added to solution 1 just prior to use)

Solution 2

2 ml NaOH 1N

1 ml 10% SDS

7 ml H₂O

Solution 3

60 ml of 5 M potassium acetate

11.5 ml of glacial acetic acid

28.5 ml of H₂O

A single bacterial colony was transferred into 10 ml of LB broth containing 50 mg/l of ampicillin and incubated overnight at 37°C with vigorous shaking.

1 ml of cells were centrifuged at 12,000 g for 1 min and the pellet resuspended in 100 ul of ice-cold solution 1 by vortexing vigorously. This was left to

stand at room temperature for 5 mins, after which 200 ul of solution 2 was added and mixed by inverting the tube 3-4 times, before leaving on ice for 5 mins. 150 ul of ice cold solution 3 was added and vortexed gently for 10 secs, then left on ice for 5 mins before centrifuging at 12,000 g for 5 mins. The supernatant (S/N) was subsequently transferred to a fresh tube, extracted with an equal volume of phenol-chloroform and precipitated with 2 volumes of ethanol at room temperature for 2 mins. This was then centrifuged again at 12,000 g for 5 mins, and the S/N removed by gentle aspiration.

The pellet was then washed with 1 ml of 70% ethanol at 4°C, the S/N removed and the pellet of nucleic acid air-dried for 10 mins. The nucleic acids were redissolved in 50 ul of TE containing RNAase (20 ug/ml) and stored at -20°C.

Restriction enzyme digest of the isolated plasmid pJIT134 and separation of the 2 fragments produced.

The DNA pellet was resuspended in 22 ul of distilled water, 2.5 ul of the appropriate buffer and 1 ul of *Eco* R1 and digested for 3 hours at 37°C. The DNA was then phenol-chloroform extracted, ethanol precipitated and resuspended in 22 ul of distilled water, 2.5 ul of the appropriate buffer and 1 ul of *Hind* III and again digested for 3 hours at 37°C. After digestion, 5 ul of loading buffer was added to the *Hind* III assay mixture and loaded onto an agarose (1.8 g in 200 ml TE) mini-

gel. The gel was allowed to run at 100 v for 1 hour. A Lambda *Hind* III-*Eco* RI digest was run as a molecular weight marker in the end well of the gel.

Electroelution of the appropriate DNA band from the gel.

After the gel had been run for 1 hour, it was stained with ethidium bromide and examined under UV light and the piece of agarose containing the appropriate band was removed with a razor blade.

The piece of gel was placed into dialysis tubing which had been soaked in TE, and 200 ul of 1xTE was added to the tubing which was sealed with a clip.

The tubing was immersed in 1xTE in a mini-gel electrophoresis tank and 100 v passed through the bag for 1/2 hour.

The TE containing the DNA was placed into an Eppendorf tube and extracted with 1 volume of phenol-chloroform. Precipitation of the DNA then occurred with 1/10 volume of 3 M sodium acetate and 3 volumes of ethanol.

The DNA pellet obtained after centrifugation was resuspended in 20 ul of water.

Oligolabelling of the probe.

The DNA obtained above was denatured by boiling in an eppendorf tube and labelled by adding (in the following order) 3 ul oligonucleotides (dATP, dTTP, dGTP), 2 ul

bovine serum albumin, 5 ul gamma ^{32}P dCTP and 1ul Klenow.

The reaction was allowed to continue at room temperature for 5 hours after which time the reaction mixture was flushed through a "Sephadex" column with 12x 100 ul amounts of TE. Each 100 ul added to the column was collected at the bottom in an Eppendorf tube and each tube monitored for radioactivity. Where the first peak in radioactivity occurred, the 3 best tubes were pooled and the probe was alkali-denatured (100 ul NaOH, 100 ul HCL and 100 ul Tris pH 8).

Hybridisation of the labelled probe to nitrocellulose.

Prehybridisation solution.

2 mls of 40 x Denhardts
3 mls of 20 x SSC
400 ul boiled calf thymus DNA
14.6 mls of H_2O

The baked filter was placed in a bag with the prehybridisation solution and the denatured labelled probe was injected. This was left overnight in a water bath at 68°C . The filter was then washed by pouring 0.1 x SSC and 0.5 % SDS into the bag. This was carried out twice (the radioactive waste was discarded) after which the filter was removed and placed in a sandwich box and washed further at 65°C whilst shaking. This was carried out 3 times.

The filter was air dried and placed between blotting paper and put in a cassette with x-ray film (Kod XAR-2). This was left at -70°C for at least 3 days.

2) The NPT II assay procedure for plant material.

The assay procedure described below is according to McDonnell et al. (1987).

Extraction buffer (2 x stock).

Glycerol	2.5 ml
Tris-HCL 0.5 M	2.5 ml
Beta-mercaptoethanol	1.0 ml
SDS	20.0 mg
Final volume in H ₂ O	10.0 ml

Reaction buffer (5 x stock).

Tris	20.183 g
MgCl	21.348 g
NH ₄ Cl	53.490 g

Dissolved in 300 ml H₂O then titrated to pH 7.1 with 1 M malic acid and the total volume raised to 500 ml with H₂O.

Assay mixture

Reaction buffer 1x	4.936 ml
ATP (10 mM stock)	5.0 ul

Gamma ^{32}P -ATP (10 uCi/ul stock)	1.5 ul
Neomycin (22 mM stock)	7.0 ul
NaF (1 M stock)	50.0 ul

Procedure

Plant tissue was ground in liquid nitrogen, extraction buffer was added depending on the amount of tissue available, and grinding continued. The ground sample was transferred into an Eppendorf tube and centrifuged for 5 mins at 4°C and the supernatant decanted into a new tube.

15 ul of extract and 15 ul of assay mixture were mixed well in an Eppendorf tube and incubated at 37°C for 30 mins. During the incubation period, Whatman P 81 paper was soaked in a solution of 20 mM ATP and 100 mM of pyrophosphate and dried. This was carried out to eliminate non specific binding to the paper and background in the autoradiogram.

When the incubation was completed, this tube was centrifuged for 5 mins at 10,000 rpm and 20 ul of each sample was spotted onto dried P 81 paper.

After the spots had dried, the paper was washed for 2 mins in 10 mM sodium phosphate buffer pH 7.5 at 80°C. The blot was then washed in 10 mM buffer at room temperature for 10 mins. This was repeated 3-5 times, after which the blot was dried and exposed to X-ray film overnight.

3) The GUS assay procedures for plant material.

Fluorogenic GUS Assay.

The fluorogenic GUS assay is based on the conversion of a non-fluorescent substrate, in this case 4-methyl umbelliferyl glucuronide (MUG), to a fluorescent product 4-methyl umbelliferone (MU). As the enzyme does not occur naturally in soft fruit, extracts from non-transformed plants are unable to break down the substrate to produce a fluorescent product. The GUS assay procedure was carried out according to R.A. Jefferson (1987a,b).

Procedure

<u>Extraction buffer</u>	Volume (ml)
Na ₂ HPO ₄ 1 Molar (M) pH 7.0	25
NaH ₂ PO ₄ 1 M	25
Na ₂ EDTA 0.5 M	20
Sarcosyl 10%	10
Triton X-100 10%	10
Beta-mercaptoethanol (14.4 M)	0.7

Substrate

4-Methyl umbelliferyl B-D-glucuronidase
1 mM in 270 ul of extraction buffer

Stop buffer

Na_2CO_3 0.2 M dissolve in distilled water

Preparation of plant material.

The GUS assay procedure was carried out as described by Jefferson (1987a,b). Briefly, approximately 0.1 g of plant material from each plant to be assayed, was ground in 350 μl of extraction buffer in an Eppendorf tube. This was then spun at 10,000 rpm and the supernatant decanted and put on ice. The same plant material was ground in a further 350 μl of extraction buffer, spun and decanted into the same tube as before. This was done for all the material tested using new grinding rods and Eppendorf tubes for each different plant sample.

Before the reaction was initiated, both substrate and extract were brought to a temperature of 37°C in a water bath.

To start the reaction 50 μl of substrate was added to 350 μl of the extract in the water bath.

At time intervals, samples were taken and examined for fluorescence. This was done by removing 100 μl of the reaction mixture and adding to 900 μl of Na_2CO_3 . The addition to Na_2CO_3 serves to stop the reaction and also to maximise the fluorescence of the product, 4-MU.

Fluorescence due to 4-methyl umbelliferone (4-MU) can easily be detected using a simple UV lamp at a

wavelength of 365 nm. In my experiment I found that if the substrate was freshly prepared for each assay, then fluorescence at zero time (MU in MUG) could be completely eliminated as observed by the eye under a simple UV lamp. Fluorescence can also be quantified using a fluorimeter calibrated to known standards.

A non-inoculated plant was used as a control to determine any background beta-glucuronidase activity in the plant material. Using non-transformed plants and long overnight assays, no fluorescence was detectable visually and therefore no intrinsic beta-glucuronidase enzyme activity exists which will give positive results in an assay. Also, no background fluorescence was produced from compounds in the plant material not due to the GUS enzyme.

Because Agrobacterium containing the plasmid PBI121 showed significant GUS activity (due to read-through transcription from the lacZ transcript of pBIN19 into the GUS coding region, followed by translation initiation at the GUS initiator), care has to be taken that plant material to be assayed is sterile. This was done by grinding up some of the plant material to be assayed in liquid LB and plating on LB agar.

Histochemical localisation of B-Glucuronidase activity in leaf tissue.

The substrate used for histochemical localisation of beta-glucuronidase activity in tissues and cells is 5-

bromo-4-chloro-3-indolyl glucuronide (X-Gluc). This substrate gives a blue precipitate at the site of enzyme activity. The product of glucuronidase action on X-Gluc is not coloured. The indoxyl derivative produced must undergo an oxidative dimerization to form the insoluble and highly coloured indigo dye. This dimerisation is stimulated by atmospheric oxygen, and can be greatly enhanced by using an oxidation catalyst such as K^+ ferricyanide/ferrocyanide mixture (Pearse 1976).

Procedure

X-Gluc Stain

5 mg of X-Gluc was dissolved in 50 μ l dimethyl formamide and the volume made up to 10 ml by the addition of 50 mM $NaPO_4$, pH 7.0.

X-Gluc staining was carried out on leaf tissue from inoculated regenerated plants. 4 methods of fixation of the tissue were examined and non fixed tissue was also stained.

Methods of Fixation

For fixation, plant material is placed in one of the following solutions for the time given.

- 1) Formalin acetic acid 70% 6 hours.
- 2) Glutaraldehyde 5% 6 hours.

- 3) 1:1:1 solution of ethanol, lactic acid and chloroform
12 hours.
- 4) Chloros 5% 5 mins.
- 5) Non-fixed tissue.

Staining of fixed tissue.

After leaf tissue had been fixed in one of the above solutions, the material was rinsed in 50 mM NaH_2PO_4 pH 6.0.

Once rinsed, the plant material was then placed in a solution of X-Gluc as described above and left to stain at room temperature for 1 hour. After staining the tissue was cleared in 70% ethanol and examined.

Section 5.1 The use of the NPT II gene in soft fruit transformation.

In order for the NPT II gene encoding resistance to the antibiotics kanamycin, neomycin and G-418 to be used as a selectable marker of transformation, obvious differences have to occur between plants transformed with the NPT II gene and non-transformed plants on selectable media.

To determine if this gene would be of use as a selectable marker for transformation of soft fruit species, an initial investigation was carried out to examine the effects of the antibiotic kanamycin on non-transformed genotypes of Rubus, Ribes, Vaccinium and Fragaria spp.

If experiments demonstrated this antibiotic to have a noticeable effect on the non-transformed plants, then this gene would be of use as a selectable marker of transformed plants (assuming the NPT II gene was expressed in transformed plants).

Experiment 5.1.1.

The effect of the antibiotic kanamycin on Fragaria Rubus, Ribes and Vaccinium spp.

In the following experiment, the antibiotic kanamycin was used alone and in conjunction with the antibiotic carbenicillin (routinely used to remove Agrobacterium contamination from plant material) in

tissue culture multiplication medium. This experiment was also carried out on Tayberry and strawberry cv. Rapella plants inoculated with 516, a wild type Agrobacterium isolate (not containing a NPT II gene between the T-DNA borders), to examine any effect this might have on the plant growth in antibiotic medium.

Plant Genotypes: Rubus Genotypes

Tayberry

8242E6

Loch Ness

Fragaria Genotypes

Rapella

Vaccinium Genotypes

North Country

Ribes Genotypes

Ben More

Number of plants: 20 plants per genotype

Bacterial isolates: 516

Media: Raspberry multiplication medium

Strawberry multiplication medium

Woody Plant medium

Blackcurrant medium

(media compositions are listed in chapters 3 and 4).

Antibiotics: 0, 25, 50, 75, 100, 125 and 150 mg/l of both kanamycin and carbenicillin. Each concentration of one antibiotic was used against each concentration of the other.

Experimental procedure:

Preparation of non-inoculated plant material:

Plant material for the experiment was obtained from the stock cultures on multiplication medium, at week 4 of the tissue culture cycle and placed immediately onto antibiotic-containing medium.

Preparation of inoculated plant material:

Inoculation of plantlets from the Rubus cv. Tayberry and the Fragaria cv. Rapella was conducted as described in chapter 2. Briefly, the inoculum was grown up overnight in LB at 28°C in a shaking water bath, centrifuged and resuspended in 30 mls liquid NA medium. Plant material was obtained from the stock cultures at week 4 of culture cycle, wounded once and dipped into a suspension of 516 for 20 mins. After inoculation, the plants were placed on moist filter paper discs to incubate for 4 days after which time the plant material was dipped into a solution of 200 mg/l carbenicillin and rinsed in distilled water to remove the Agrobacterium and placed onto antibiotic-containing medium as above.

Controls of Tayberry were also established that had been treated in the same way as the inoculated plants only without the inoculum, to ensure that if differences did occur between inoculated and non-inoculated plants then they were not due to the above treatment of the plant material.

Results were recorded after a period of 5 weeks.

Results Experiment 5.1.1.

The tables below indicate the numbers of plants out of a total of 20 which whitened on the antibiotic media.

Rubus spp.

Table 5.1.1a. The effect of kanamycin alone and in conjunction with carbenicillin on Tayberry.

		CARBENICILLIN mg/l.						
		0	25	50	75	100	125	150
K								
A	0	0	0	0	0	0	0	0
N	25	4	5	5	6	5	5	7
A	50	20	20	18	20	20	20	20
M	75	20	20	20	20	20	20	20
Y	100	20	20	20	20	20	20	20
C	125	20	20	20	20	20	20	20
I	150	20	20	20	20	20	20	20
N								

Table 5.1.1b. The effect of the antibiotics kanamycin and carbenicillin on Tayberry plants inoculated with Agrobacterium isolate 516.

		CARBENICILLIN mg/l.						
		0	25	50	75	100	125	150
K								
A	0	0	0	0	0	0	0	0
N	25	0	0	0	4	6	5	8
A	50	7	6	9	10	9	10	12
M	75	20	20	20	20	20	20	20
Y	100	20	20	20	20	20	20	20
C	125	20	20	20	20	20	20	20
I	150	20	20	20	20	20	20	20
N								

Table 5.1.1c. The effect of the antibiotics kanamycin and carbenicillin on Tayberry control plants.

		CARBENICILLIN mg/l.						
		0	25	50	75	100	125	150
K								
A	0	0	0	0	0	0	0	0
N	25	2	4	5	7	5	5	7
A	50	20	19	20	20	20	20	20
M	75	20	20	20	20	20	20	20
Y	100	20	20	20	20	20	20	20
C	125	20	20	20	20	20	20	20
I	150	20	20	20	20	20	20	20
N								

Fragaria spp.

Table 5.1.1d. The effect of the antibiotics kanamycin and carbenicillin on Rapella.

		CARBENICILLIN mg/l.						
		0	25	50	75	100	125	150
K								
A	0	0	0	0	0	0	0	0
N	25	9	19	16	20	16	19	18
A	50	17	19	20	17	20	20	20
M	75	19	20	20	20	20	19	18
Y	100	20	20	20	20	20	20	20
C	125	20	20	20	20	20	20	20
I	150	20	20	20	20	20	20	20
N								

Table 5.1.1e. The effect of the antibiotics kanamycin and carbenicillin on Rapella plants inoculated with Agrobacterium isolate 516.

		CARBENICILLIN mg/l.						
		0	25	50	75	100	125	150
K								
A	0	0	0	0	0	0	0	0
N	25	7	14	11	12	15	13	18
A	50	15	18	20	14	17	20	20
M	75	18	16	18	20	19	20	20
Y	100	20	20	20	20	20	20	20
C	125	20	20	20	20	20	20	20
I	150	20	20	20	20	20	20	20
N								

Vaccinium spp.

Table 5.1.1f. The effect of the antibiotics kanamycin and carbenicillin on North Country.

		CARBENICILLIN mg/l.						
		0	25	50	75	100	125	150
K								
A	0	0	0	0	0	0	0	0
N	25	11	12	12	10	14	13	13
A	50	14	14	15	17	16	20	19
M	75	17	15	16	20	15	19	18
Y	100	18	17	18	20	18	20	20
C	125	20	20	20	20	20	20	20
I	150	20	20	20	20	20	20	20
N								

Ribes spp.

Table 5.1.1g. The effect of the antibiotics kanamycin and carbenicillin on Ben More.

		CARBENICILLIN mg/l.						
		0	25	50	75	100	125	150
K								
A	0	0	0	0	0	0	0	0
N	25	16	20	17	14	20	19	20
A	50	17	17	19	17	20	20	19
M	75	20	20	20	20	20	20	20
Y	100	20	20	20	20	20	20	20
C	125	20	20	20	20	20	20	20
I	150	20	20	20	20	20	20	20
N								

In the above experiments, whitening was taken to be any patch of white material occurring on the plants, as no whitening is observed normally.

As can be seen from the tables above, kanamycin has a whitening effect on Tayberry plants which tends to increase with the level of kanamycin in the medium until

at 50 mg/l for non-inoculated plants, and 75 mg/l for inoculated plants, whitening occurred on all the plant material.

Whitening of all plants of Rapella (both inoculated and non-inoculated) and North Country at a given concentration did not occur until 100 mg/l of kanamycin was incorporated in the medium. With Ben More, whitening of the majority of plants occurred at the lowest kanamycin concentration (25 mg/l); however, whitening of all plants did not occur until a concentration of 75 mg/l of kanamycin was present.

That the whitening effect was not due to carbenicillin can be seen from the tables where no whitening occurred when there was no kanamycin in the medium, regardless of the amount of carbenicillin used. In contrast, whitening occurred in plant material on kanamycin-containing medium, in the absence of carbenicillin.

Downward trends can be seen in the tables with the amount of plants affected increasing as the amount of kanamycin increased.

Stunting of the growth of the plant material occurred due to the combined effects of high concentrations of both antibiotics. The blueberry North Country and the blackcurrant Ben More appeared to be particularly sensitive to high concentrations of both antibiotics, with distortion of growth and a reduction in branching occurring as well as stunting.

For some reason, the Agrobacterium isolate 516 appeared to lessen the effect of kanamycin, at concentrations up to and including 50 mg/l kanamycin on Tayberry plants.

Unlike the Tayberry whitening experiment, no obvious difference was obtained from inoculated and non-inoculated Rapella plants.

Although whitening of plant material was detected to some extent in all plant material from 25 mg/l of kanamycin, the plant material, especially of Rubus and Fragaria, still grew normally. Only when high levels of both kanamycin and carbenicillin were incorporated into the medium did stunting and some distortion of the plant material occur.

This first experiment demonstrated that kanamycin at a concentration of 100 mg/l had a very noticeable whitening effect on all the non transformed plant material tested.

This effect caused by kanamycin on non transformed plant material indicated that NPT II would be of use as a marker of transformation.

The following figure (5.1) illustrates the effect of kanamycin on plant material in tissue culture. Jar A on the left of the photograph contains media with 50 mg/l kanamycin, the jar on the right (B) contains no kanamycin.

Fig. 5.1



A

B

The growth of the hybrid berry Tayberry on medium RM (B) and medium RM containing 50 mg/l of the antibiotic kanamycin (A).

Before inoculation of plant material with Agrobacterium containing a binary vector with the kanamycin marker gene (to effect transfer of this gene into the plant material) was carried out, a second "preliminary" experiment was carried out to determine the effect of inoculation with Agrobacterium on plant regeneration.

Experiment 5.1.2.

The effect of Agrobacterium on the regeneration of explant material.

Plant Genotypes: Tayberry
Loch Ness

Bacterial isolates: LBA4404
Ar9402

Media: NA
Liquid NA without hormones or sucrose.

Hormones: 0.2 mg/l BAP and 0.2 mg/l 2,4-D
0.1 mg/l IBA and 2 mg/l BAP

Experimental procedure: Explant material for inoculation and preparation of the inoculum were carried out as described in section 5.0.

Briefly, the inoculum was prepared by growing the isolates overnight in the appropriate broth containing the appropriate antibiotic.

After an overnight incubation, the culture was centrifuged, washed in liquid NA medium, re-centrifuged and finally resuspended in 30 mls of liquid NA medium. The Agrobacterium suspensions were poured into 9 cm diameter petri dishes into which the explant material was placed for inoculation. The explant material was left in the Agrobacterium suspension for 20 mins after which time the plant material was removed and placed onto filter paper discs moistened with liquid plant medium in sterile petri dishes and left to incubate for 12 hours.

After 12 hours, the plant material was dipped into 400 mg/l carbenicillin and placed onto regeneration medium (NA with 0.2 mg/l BAP and 0.2 mg/l 2,4-D for internodal segments and NA with 0.1 mg/l IBA and 2 mg/l BAP for leaf discs).

Non-inoculated controls were prepared as above but without Agrobacterium.

Results Experiment 5.1.2.

Table 5.1.2a. The effect of Agrobacterium isolate Ar9402 on internodal segment regeneration.

	Inoculated		Non-inoculated	
	Tayberry	Loch Ness	Tayberry	Loch Ness
No.segments	30	30	30	30
No.regen.shoots	5(17%)	2(7%)	14(47%)	12(40%)
No.regen.roots	4	2	0	0

Table 5.1.2b. The effect of Ar9402 on leaf disc regeneration.

	Inoculated		Non-inoculated	
	Tayberry	Loch Ness	Tayberry	Loch Ness
No.leaf discs	30	30	30	30
No.regen.shoots	3(10%)	4(13%)	9(30%)	9(30%)
No.regen.roots	4	5	0	0

Table 5.1.2c. The effect of LBA4404 on internodal segment regeneration.

	Inoculated		Non-inoculated	
	Tayberry	Loch Ness	Tayberry	Loch Ness
No.segments	30	30	30	30
No.regen.	9(30%)	8(27%)	12(40%)	10(33%)

Table 5.1.2d. The effect of LBA4404 on leaf disc regeneration.

	Inoculated		Non-inoculated	
	Tayberry	Loch Ness	Tayberry	Loch Ness
No.leaf discs	30	30	30	30
No.regen.	6(20%)	8(27%)	10(33%)	9(30%)

The above results demonstrate a reduction in the regeneration potential of internodal segments due to transformation by A. rhizogenes ($P < 0.01$). Ar9402 is a wild-type isolate, and thus the transfer of genes involved in the synthesis of hormones was probably responsible for the decline in regeneration by upsetting the hormone balance. Of the plants regenerated after internodal segment inoculation with Ar9402, 4/5 Tayberries and 2/2 Loch Ness plants demonstrated the presence of large white hairy roots not seen on any of the LBA4404 inoculated or non-inoculated plants. With leaf disc inoculation, 2/3 plants of Tayberry and 3/5 Loch Ness demonstrated large hairy roots.

A reduction in regeneration of the controls, compared to that found in chapter 3, was also observed, probably due to the inoculation procedure rather than the inoculum as there was no inoculum present in the controls.

LBA4404-inoculated plants and the controls did not show any significant differences, again suggesting the actual procedure is responsible for the reduction in regeneration.

Following completion of the 2 preliminary investigations, experiments were set up to determine if Agrobacterium transfer of the NPT II gene would occur into soft fruit.

Experiment 5.1.3.

The inoculation of Sunberry explants with Agrobacterium containing Bin 19.

Plant Genotypes: Sunberry (initially chosen as this genotype had the highest rate of success with leaf disc regeneration).

Bacterial isolates: LBA4404:Bin19

Ar9402:Bin19

No. explants: 60 discs and 60 internodal segments per treatment.

Media composition: Liquid NA

Solid NA

Hormone combinations: 0.1 mg/l IBA and 2 mg/l BAP

0.2 mg/l BAP and 0.2 mg/l 2,4-D

Antibiotics: Carbenicillin (used to control Agrobacterium contamination) and kanamycin (used to

select for transformants).

Experimental procedure: 2x10 ml cultures of each of the above bacteria were grown up overnight, centrifuged as described above and each culture resuspended in 30 mls of liquid NA.

Leaf discs were obtained as described previously, and inoculated by dipping in a 30 ml suspension of LBA4404:Bin19 or Ar9402:Bin19, contained within a sterile 9 cm diameter petri dish for 20 minutes. When removed from the Agrobacterium suspension, the discs were placed onto filter paper discs moistened with NA medium and left again in sterile petri dishes to incubate for 12 hours. After incubation, the discs were removed and placed onto medium NA, containing 0.1 mg/l of IBA, 2 mg/l BAP and 100 mg/l of carbenicillin.

The discs were left on this medium for 4 days after which time they were placed onto fresh medium as above, but containing in addition 75 mg/l of kanamycin to select for transformants.

The above procedure was repeated with internodal segments the only difference being the use of the hormone combination 0.2 mg/l BAP and 0.2 mg/l 2,4-D in place of the IBA and BAP combination used for the leaf discs.

Controls were set up for both leaf discs and internodal segments, and these were subjected to

exactly the same conditions as above except there was no Agrobacterium in the liquid NA medium in which they were dipped for 20 mins.

Results Experiment 5.1.3.

In this experiment no results were obtained as 100 mg/l of carbenicillin in the medium could not contain the Agrobacterium which completely overran the plant material. Death of all explants occurred within 12 days.

This experiment was repeated below with minor alterations.

Experiment 5.1.4.

The inoculation of Sunberry explants with a weaker inoculum of Agrobacterium.

Plant Genotype: Sunberry

Bacterial isolates: LBA4404:Bin19

Ar9402:Bin19

No. explants: 60 leaf discs and internodal segments per treatment.

Media: Liquid NA

Solid NA

Hormone combinations: 0.1 mg/l IBA and 2 mg/l BAP

0.2 mg/l BAP and 0.2 mg/l 2,4-D

Antibiotics: Carbenicillin

Kanamycin

Experimental procedure: This experiment was carried out as exp.5.1.3, with one modification. In exp.5.1.3, Agrobacterium overran the plant material, therefore in this experiment a weaker inoculum was used. 1x10 ml cultures of each bacterium were grown up overnight in place of 2x10 ml cultures and resuspended in 60 mls of liquid NA instead of 30 mls, and this was then divided into 2x30 ml amounts for inoculation.

Results Experiment 5.1.4.

In this experiment Agrobacterium contamination was not so much of a problem as in exp.5.1.3. Carbenicillin in the medium still did not completely control the Agrobacterium contamination and fresh medium was required every 3-4 days. After a period of 10 days however, the leaf discs were beginning to brown and by 21 days after inoculation, all the discs were dead. The internodal segments began browning around 15 days after inoculation and by 28 days, death of all the segments had occurred. The fact that the control plants also

browned suggested that the medium with the antibiotics was the cause rather than the Agrobacterium. The antibiotics were further implicated as being the cause of browning and death as the same medium without the antibiotics had previously induced leaf disc and internodal segment regeneration.

At this stage I decided to examine the effect of carbenicillin, kanamycin and a third antibiotic cefotaxime (also used to control Agrobacterium contamination) on the regeneration of leaf disc and internodal segments. The 3 experiments (5.1.5-5.1.7) involving their use were as follows.

Experiment 5.1.5.

The effect of kanamycin on the regeneration of Rubus spp.

Due to the failure of inoculated leaf discs and internodal segments to regenerate, the effect of kanamycin was examined on non inoculated plant material to determine if the presence of this antibiotic (required for the selection of transformants) was inhibiting regeneration.

Plant genotypes : Sunberry
Tayberry

No. explants: 60 leaf discs / treatment

30 internodal segments / treatment

Medium composition: NA

Hormone combinations: 0.1 mg/l IBA and 2 mg/l BAP

0.2 mg/l BAP and 0.2 mg/l 2,4-D

Antibiotic concentrations: kanamycin at 0, 25, 50
and 75 mg/l.

Experimental conditions: 60 leaf discs were placed
on medium NA containing 0.1 mg/l IBA and 2 mg/l BAP
and each of the 4 different antibiotic
concentrations.

30 internodal segments were placed onto NA with
0.2 mg/l BAP and 0.2 mg/l 2,4-D again at each
antibiotic concentration.

Regeneration conditions were as described in
chapter 3.

Results Experiment 5.1.5.

Results from the above experiment demonstrate
the devastating effect of kanamycin on explant
regeneration. Regeneration dropped from around 50% for
leaf discs and internodal segments of Sunberry and
Tayberry with 0 mg/l of kanamycin, to 0% for plant
material on 75 mg/l of kanamycin.

Table 5.1.5a. The effect of kanamycin on the regeneration of Sunberry and Tayberry leaf discs.

	Kanamycin concentration mg/l.			
	0	25	50	75
Sunberry				
No. discs	60	60	60	60
No. callus	11	2	0	0
No. regen	35(58%)	0	0	0
Tayberry				
No. discs	60	60	60	60
No. callus	7	0	0	0
No. regen.	29(48%)	0	0	0

Table 5.1.5b. The effect of kanamycin on the regeneration of Sunberry and Tayberry segments.

	Kanamycin concentration mg/l.			
	0	25	50	75
Sunberry				
No. segs.	30	30	30	30
No. callus	10	5	4	0
No. regen.	12(40%)	0	1(3.3%)	0
Tayberry				
No. segs.	30	30	30	30
No. callus	5	2	0	0
No. regen.	15(50%)	0	0	0

Death of all explants on the kanamycin medium occurred within 14 days.

Experiment 5.1.6.

The effect of carbenicillin on the regeneration of Rubus spp

In order to determine whether the level of carbenicillin was contributing to the browning and

death of the explant material, the effect of 5 different concentrations of this antibiotic was examined here.

Plant Genotypes: Sunberry
Tayberry
Loch Ness

No. explants: 20 leaf discs and internodal segments
/ treatment.

Medium composition: NA

Hormones: 0.1 mg/l IBA and 2 mg/l BAP.
0.2 mg/l BAP and 0.2 mg/l 2,4-D

Antibiotic concentration: Carbenicillin at 0, 50,
100, 250 and 500 mg/l.

Experimental conditions: 20 discs and internodal segments of the above genotypes were placed on the above medium at each concentration of carbenicillin. Regeneration conditions were as described in chapter 3.

Results Experiment 5.1.6.

The antibiotic carbenicillin, generally used to remove Agrobacterium contamination in tissue culture, was found

to be inhibitory to plant regeneration at the level incorporated into regeneration medium (100 mg/l).

Table 5.1.6a. The effect of carbenicillin on leaf discs of Sunberry, Tayberry and Loch Ness.

Carbenicillin concentration mg/l.					
	0	50	100	250	500
Sunberry					
No. discs	20	20	20	20	20
No. callus	14	5	2	0	0
No. regen.	10(50%)	2(10%)	1(5%)	0	0
Tayberry					
No. discs	20	20	20	20	20
No. callus	10	3	0	0	0
No. regen.	7(35%)	1(5%)	0	0	0
Loch Ness					
No. discs	20	20	20	20	20
No. callus	13	4	0	0	0
No. regen.	9(45%)	2(10%)	0	0	0

Table 5.1.6b. The effect of carbenicillin on internodal segments of Sunberry, Tayberry and Loch Ness.

Carbenicillin concentration mg/l.					
	0	50	100	250	500
Sunberry					
No. segments	20	20	20	20	20
No. callus	16	7	3	0	0
No. regen.	11(55%)	4(20%)	0	0	0
Tayberry					
No. segments	20	20	20	20	20
No. callus	14	4	5	0	0
No. regen.	9(45%)	1(5%)	2(10%)	0	0
Loch Ness					
No. segments	20	20	20	20	20
No. callus	17	7	4	0	0
No. regen.	12(60%)	5(25%)	2(10%)	0	0

An alternative antibiotic was desirable. The antibiotic cefotaxime was examined in exp.5.1.7 as an alternative to carbenicillin.

Experiment 5.1.7.

The effect of the antibiotic cefotaxime on the regeneration of Sunberry leaf discs and internodal segments.

Plant Genotypes: Sunberry

Medium: NA

Hormones Used: 0.1 mg/l IBA and 2 mg/l BAP.

Antibiotic concentrations: 0, 100 and 250 mg/l.

Experimental conditions: 30 discs and internodal segments of Sunberry were placed onto medium containing the 3 cefotaxime concentrations.

Again regeneration conditions were as in chapter 3.

Results Experiment 5.1.7.

On the cefotaxime medium, no organised shoot or root regeneration occurred as shown in table 5.1.7; however, what appeared to be malformed structures did form at 50 mg/l, 100 mg/l and 250 mg/l.

Table 5.1.7. The effect of cefotaxime on Sunberry leaf disc and internodal segment regeneration.

	cefotaxime concentration mg/l			
	0	50	100	250
Sunberry				
No. discs	30	30	30	30
No. callus	18	9	10	2
No. regen.	14 (47%)	0	0	0
No. segments	30	30	30	30
No. callus	23	11	7	4
No. regn.	17 (57%)	0	0	0

In the light of the results from the above antibiotic experiments, which clearly demonstrated the severe effect of antibiotics on plant regeneration, it was decided to reduce the length of time the explants were in contact with antibiotic medium.

Experiment 5.1.8.

The inoculation of Sunberry leaf discs and internodal segments with LBA4404:Bin 19 and the effect on inoculated explants of reducing the time spent on antibiotic medium.

Plant genotype: Sunberry

Bacterial isolate: LBA4404:Bin 19

No. explants: 30 leaf discs and internodal segments
/ treatment.

Media: NA

Hormones: 0.1 mg/l IBA and 2 mg/l BAP

0.2 mg/l BAP and 0.2 mg/l 2,4-D

Antibiotics: Carbenicillin

Kanamycin

Experimental procedure: Inoculation was carried out as in experiment 5.1.2, using the weaker inoculum. After incubation for 12 hours, the explants were placed onto media containing 100 mg/l of carbenicillin and left for 3 days, after which time the discs were placed onto selection medium containing 100 mg/l carbenicillin and 75 mg/l kanamycin. Groups of 30 explants were left for varying periods of time (2,3,4,5 and 10 days) on this medium, after which time they were removed and placed onto medium without any antibiotics.

Results Experiment 5.1.8.

The results from this experiment demonstrated the severe effect of antibiotic selection medium on explant survival and regeneration, even when explants were in contact with the medium for only a short period of time.

Table 5.1.8. The ability of explants to regenerate after varying times on selection medium.

	Days on kanamycin selection medium									
	2		3		4		5		10	
	Inoc	C	Inoc	C	Inoc	C	Inoc	C	Inoc	C
No.leaf disc	30	30	30	30	30	30	30	30	30	30
No. died	29	27	27	30	30	30	30	30	30	30
No. regen.	0	0	0	0	0	0	0	0	0	0
No.inter.segm	30	30	30	30	30	30	30	30	30	30
No. died	22	25	27	29	26	30	30	30	30	30
No. regen.	1	0	0	0	0	0	0	0	0	0

The following experiment examined the effect of antibiotic medium when used to control contamination during only the first 4 days after inoculation.

Experiment 5.1.9.

The inoculation of Tayberry leaf discs and internodal segments with LBA4404:Bin 19 and Ar9402:Bin 19 and the regeneration of those segments in the absence of selection.

Plant genotype: Tayberry

Bacterial isolate: LBA4404:Bin 19

Ar9402:Bin 19

No. explants: 60 leaf discs and internodal segments/ treatment

Media: NA

Hormones: 0.1 mg/l IBA and 2 mg/l BAP

0.2 mg/l BAP and 0.2 mg/l 2,4-D

Antibiotics: Kanamycin

Carbenicillin

Cefotaxime

Experimental procedure: 120 leaf discs and 120 internodal segments were inoculated with both LBA4404:Bin 19 and Ar9402:Bin 19. The inoculation was carried out as in exp.5.1.3, with the weaker inoculum used in exp.5.1.4 (overnight culture diluted in 60 mls liquid medium and divided between 2 petri dishes rather than diluted in 30 mls and placed into 1 petri dish, as in exp.5.1.1).

After the 12 hour incubation on filter paper, the 120 leaf discs and internodal segments were divided into groups of 60 and placed on NA containing the appropriate hormones for regeneration plus either 100 mg/l carbenicillin or 250 mg/l cefotaxime.

After 4 days the explants were placed onto the same medium minus the antibiotics. After 21 days (by which time a few of the the explants were showing signs of regeneration), the explants were placed again onto the same medium to which 75 mg/l

of kanamycin had been added to select for transformants.

Results Experiment 5.1.9.

Although this experiment did yield regenerants, the number of plantlets produced from inoculated explants was only 28/480 or 6.0% (38/240 or 15.8% plantlets regenerated from non inoculated explants).

Table 5.1.9a. The results of regenerating inoculated Tayberry internodal segments in the absence of selection.

	Ar9402		LBA4404		Non-Inoc.	
	cef	carb	cef	carb	cef	carb
No. segments	60	60	60	60	60	60
Died	48	27	40	21	32	17
Callus only	11	19	20	21	14	19
No. Regen.	1(2%)	3(5%)	0	9(15%)	7(12%)	12(20%)
No.shoots	1	6	0	12	11	16
Died	1	3	0	5	3	0
Whitened	0	2	0	4	8	16
Green	0	1	0	3	0	0

Table 5.1.9b. The results of regenerating inoculated Tayberry leaf discs in the absence of selection.

	Ar9402		LBA4404		Non-Inoc.	
	cef	carb	cef	carb	cef	carb
No. discs	60	60	60	60	60	60
Died	55	38	49	31	52	37
Callus only	5	14	11	19	2	9
No. Regen.	0	3(5%)	0	6(10%)	3(5%)	8(13%)
No. shoots	0	3	0	6	3	8
Died	0	1	0	0	0	1
Whitened	0	1	0	4	3	7
Green	0	1	0	2	0	0

The condition of the regenerants from this experiment was also very poor, especially when placed onto the kanamycin selection medium. Once regenerated, no controls remained green on kanamycin, 10/10 or 100% leaf discs and 24/24 or 100% internodal segments whitened, whereas with the inoculated plants 3/8 or 38% of plantlets which regenerated from leaf discs and 4/10 or 40% of plantlets which regenerated from internodal segments remained green.

The antibiotic cefotaxime had a much more severe effect on plant regeneration and its use was abandoned in all further experiments.

Once placed onto medium without antibiotics, Agrobacterium contamination returned and plant material, therefore, required dips into the appropriate

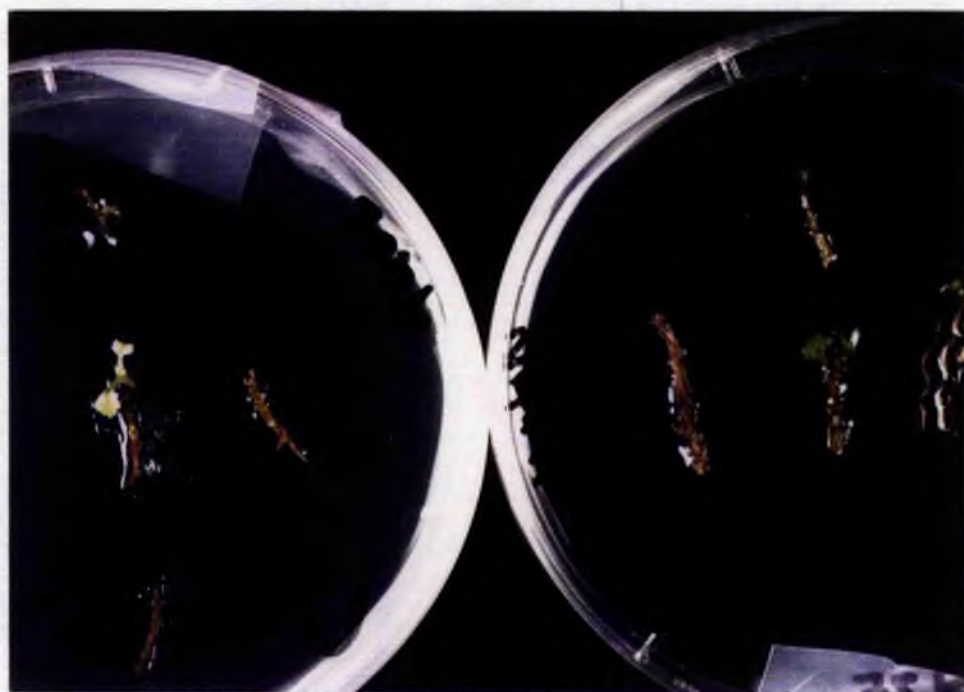
antibiotic. As the presence of antibiotics in the medium for 4 days failed to clean up Agrobacterium contamination and still appeared to affect badly regeneration, it was decided to abandon the use of antibiotics in the regeneration medium.

The next experiment examines the use of antibiotic dips on Agrobacterium contamination and their effect on plant regeneration. The inoculated regenerated plants which remained green on kanamycin medium were grown on for assay (the conditions of which are described in section 5.0). This took some time as the plantlets were very slow-growing, even when removed from the antibiotic containing medium onto which regenerated plantlets were placed to remove Agrobacterium contamination.

The following figure (5.2) shows non-inoculated (A) and inoculated (B) internodal segments placed onto medium containing 75 mg/l kanamycin after regeneration had been initiated.

The control plantlets (A) on the left have whitened due to the effect of kanamycin whereas the inoculated ones on the right (B) have remained green.

Fig. 5.2



A

B

The effect of 75 mg/l of the antibiotic kanamycin on regenerating plantlets of non-inoculated (A) and inoculated (B) explants. The plantlets regenerating from non-inoculated explants are whitening in response to the presence of kanamycin whereas the inoculated plantlets remain green due to the action of the NPT II gene product showing them to be transformed.

Experiment 5.1.10.

The inoculation of 4 Rubus genotypes with LBA4404:BIN 19 and the use of antibiotic dips to remove Agrobacterium contamination.

Plant genotypes: Sunberry
Tayberry
Loch Ness
8242E6

Bacterial isolate: LBA4404:Bin 19

No. explants: 30 discs and segments / 4 genotypes /
2 treatments

Media: NA

Hormones: 0.1 mg/l IBA and 2 mg/l BAP
0.2 mg/l BAP and 0.2 mg/l 2,4-D

Antibiotics: Carbenicillin at 200 and 400 mg/l for
dips. Kanamycin in medium 75 mg/l.

Experimental procedure: Inoculation was carried out as exp.5.1.3 using the weak inoculum exp.5.1.4. After the overnight incubation on moist filter paper discs, the plant material was placed onto the appropriate regeneration medium containing no

antibiotics. When Agrobacterium contamination became visible (after 2 days on the medium) half of the explants were dipped into 200 mg/l of carbenicillin and half were dipped into 400 mg/l carbenicillin in an attempt to clean up the contamination. After dipping into the carbenicillin for 5 minutes, the explants were then rinsed in sterile distilled water and dried on sterile filter paper discs before being placed onto fresh medium.

Only when plant regeneration had occurred were the plantlets placed onto medium containing 75 mg/l kanamycin.

Results Experiment 5.1.10.

Carbenicillin at 400 mg/l successfully controlled Agrobacterium contamination after a period of 17 days, whereas the plant material dipped into 200 mg/l of carbenicillin was lost due to becoming overrun with contamination leading to the death of the explants.

The table on the following page (5.1.10) shows the number of plantlets regenerating and the number remaining green on kanamycin containing medium.

Table 5.1.10. Regeneration of explants after inoculation and effect of kanamycin after regeneration.

	Inoculated 200 mg/l carb.		Inoculated 400 mg/l carb.		Non-Inoc. 400 mg/l carb.	
	LD	IS	LD	IS	LD	IS
Sunberry						
No. explants	30	30	30	30	30	30
Died	30	30	17	14	14	10
Callused only	0	0	8	7	3	1
No. Regen.	0	0	5(17%)	9(30%)	10(33%)	13(43%)
No. Plantlets	0	0	5	11	11	16
Died	0	0	0	3	1	3
Whitened	0	0	4	4	10	13
Green	0	0	1	4	0	0
Tayberry						
No. explants	30	30	30	30	30	30
Died	30	27	16	14	14	7
Callused only	0	3	10	8	7	9
No. Regen.	0	0	4(13%)	8(27%)	9(30%)	14(46%)
No. Plantlets	0	0	5	11	9	18
Died	0	0	1	2	0	3
Whitened	0	0	3	6	9	15
Green	0	0	1	3	0	0
Loch Ness						
No. explants	30	30	30	30	30	30
Died	29	27	20	13	10	7
Callused	1	3	5	7	10	8
No. Regen.	0	0	5(17%)	10(33%)	10(33%)	15(50%)
No. Plantlets	0	0	5	13	11	19
Died	0	0	1	2	1	2
Whitened	0	0	3	7	10	17
Green	0	0	1	4	0	0
8242E6						
No. explants	30	30	30	30	30	30
Died	30	27	17	15	10	10
Callus only	0	3	10	8	11	7
No. Regen.	0	0	3(10%)	7(23%)	9(30%)	13(43%)
Plantlets	0	0	4	11	10	15
Died	0	0	1	2	1	2
Whitened	0	0	2	5	9	13
Green	0	0	1	4	0	0

A Dot Blot assay (5.3) to examine antibiotic phosphorylation, was carried out on regenerated plants remaining green on kanamycin, non-inoculated controls were also examined.

Fig. 5.3



A B C D E F

Dot blot assay on 3 control (A-C) and 3 transformed (D-F) plants. A small amount of non-specific background activity can be detected from the controls, however the transformed plants produce a darker blot due to the action of the NPT II enzyme.

A Control hybrid berry cv. Sunberry
B Control blackcurrant cv. Ben More
C Control hybrid berry cv. Tayberry
D Tayberry
E Sunberry
F Ben More

Section 5.2: The use of the GUS gene in soft fruit transformation.

Although the NPT II gene was successfully used in the previous experiments to select for transformed plants, the dot blot assay and Southern hybridisation are time consuming and only around 15 plants can be assayed at one time. The GUS gene on the other hand has a quick, simple assay that can be carried out on a large number of plants at any one time.

The following experiments involve the inoculation of plants with the vector PBI121 containing the GUS gene.

Experiment 5.2.1

The inoculation of Rubus spp. with Agrobacterium containing the PBI121 binary vector.

Plant Genotypes: Sunberry
Loch Ness
8242E6
Tayberry

Bacterial isolates: LBA4404:PBI121
Ar9402:PBI121

Number of explants: 30 leaf discs and internodal
segments / treatment.

Media: NA

Hormones: 0.1 mg/l IBA and 2 mg/l BAP

0.2 mg/l BAP and 0.2 mg/l 2,4-D

Experimental procedure: Inoculation was carried out as exp.5.1.10 with the weaker inoculum, and incubated overnight on moist filter paper discs after which the explants were dipped into 400 mg/l carbenicillin, followed by distilled water, blotted, and placed onto the appropriate regeneration medium (this process being repeated as required). Only when regeneration had been initiated were the explants placed onto kanamycin selection medium (useful here as the binary vector PBI121 also contains the NPT II gene).

Results Experiment 5.2.1.

In this experiment no shoot regeneration occurred from leaf discs inoculated with Ar9402, and of the internodal segments only 6 out of 120 (5%) inoculated segments regenerated, producing 13 shoots. Explants inoculated with LBA4404 fared better, with 21/120 (18%) leaf discs regenerating plantlets and 33/120 (28%) internodal segments regenerating plantlets. Regeneration from leaf discs and internodal segments produced 24 and 47 shoots respectively.

From Ar9402 inoculations, 11/120 (9%) leaf discs regenerated white hairy roots and 17/120 (14%) internodal segments also regenerated hairy roots. These roots, however, could not be induced to form shoots.

Of the 13 shoots regenerated from Ar9402 inoculations, 3 were GUS positive (43% of assayed shoots, 2.5% of the total number of explants inoculated), 4 GUS negative and 6 died.

With LBA4404 21/120 (17.5%) leaf discs and 33/120 (28%) internodal segments regenerated. Of the 71 shoots which regenerated here (24 from LD and 47 from IS), 19 (39% of assayed shoots or 16% of total number of explants inoculated) were GUS positive, 30 GUS negative and 22 died before assay.

Table 5.2.1a. The regeneration ability of Sunberry explants inoculated with LBA4404:PBI121 and resulting GUS assays on regenerated plantlets.

	Ar9402:PBI		LBA4404:PBI		Controls	
	LD	IS	LD	IS	LD	IS
Sunberry						
No. explants	30	30	30	30	30	30
No. died	17	12	15	14	5	4
No. callus only	11	12	9	8	14	12
No. regen.	0	2(7%)	6(20%)	8(27%)	11(37%)	14(47%)
No. shoots	0	5	7	11	11	19
No. roots	2	4	0	0	0	0
Plantlet Assay						
No. died	0	2	3	5	0	3
GUS positive	0	1	1	3	0	0
GUS negative	0	2	3	3	11	16

Table 5.2.1b. The regeneration ability of Loch Ness explants inoculated with LBA4404:PBI121 and resulting GUS assays on regenerated plantlets.

	Ar9402:PBI		LBA4404:PBI		Controls	
	LD	IS	LD	IS	LD	IS
Loch Ness						
No. explants	30	30	30	30	30	30
No. died	18	12	19	12	7	1
No. callus only	9	11	6	8	12	15
No. regen.	0	2(7%)	5(17%)	10(33%)	11(37%)	14(47%)
No. shoots	0	5	6	15	11	17
No. regen.roots	3	5	0	0	0	0
Plantlet Assay						
No. died	0	3	1	3	3	3
GUS positive	0	1	1	4	0	0
GUS negative	0	1	4	8	8	14

Table 5.2.1c. The regeneration ability of SCRI selection 8242E6 explants inoculated with LBA4404:PBI121 and resulting GUS assays on regenerated plantlets.

	Ar9402:PBI		LBA4404:PBI		Controls	
	LD	IS	LD	IS	LD	IS
8242E6						
No. explants	30	30	30	30	30	30
No. died	9	17	16	13	8	5
No. callus	17	7	10	11	14	13
No. regen.	0	1(3%)	4(13%)	6(20%)	8(27%)	12(40%)
No. shoots	0	2	4	9	9	13
No. regen.roots	4	5	0	0	0	0
Plantlet Assay						
No. died	0	1	1	3	3	1
GUS positive	0	1	2	3	0	0
GUS negative	0	0	1	3	6	12

Table 5.2.1d. The regeneration ability of Tayberry explants inoculated with LBA4404:PBI121 and resulting GUS assays on regenerated plantlets.

	Ar9402:PBI		LBA4404:PBI		Controls	
	LD	IS	LD	IS	LD	IS
Tayberry						
No. explants	30	30	30	30	30	30
No. died	16	10	17	10	9	3
No. callus only	12	16	7	11	9	10
No. regn.	0	1(3%)	6(20%)	9(30%)	12(40%)	17(57%)
No. shoots	0	1	7	12	12	18
No. regen.roots	2	3	0	0	0	0
Plantlet Assay						
No. died	0	0	3	3	1	1
GUS positive	0	0	1	4	0	0
GUS negative	0	1	3	5	11	17

The shoots produced from inoculated explants were very slow growing compared with non inoculated explants. Non-inoculated explants produced shoots which reached a height of 2 cm within 8 weeks; however, the inoculated segments took approximately 20 weeks to reach the same height.

All GUS positive shoots remained green on kanamycin selection medium, indicating the possible co-transfer of both genes.

The use of Ar9402 was abandoned and LBA4404 used in the next experiment.

Experiment 5.2.2.

The inoculation of 4 genotypes of Rubus spp with
LBA4404:PBI121.

Plant Genotypes: Tayberry
Sunberry
Loch ness
8242E6

Bacterial isolates: LBA4404:PBI121

No. explants: 100 leaf discs and internodal
segments / genotype / treatment.

Media: NA

Hormones: 0.1 mg/l IBA and 2 mg/l BAP
0.2 mg/l BAP and 0.2 mg/l 2,4-D

Experimental conditions: As in the last
experiment.

Results Experiment 5.2.2.

The results of this experiment (shown in the tables
which follow) demonstrates the reduction in regeneration
of inoculated plantlets and the loss of a large number
of plantlets before assay. Of the 800 inoculated

explants, 170 regenerated (21%), producing 192 plantlets, and of the 200 controls, 85 regenerated (43%), producing 111 plantlets. However, of the plants which did survive to be assayed, approximately (40/96) 42% of them were GUS positive. Non-inoculated controls were always GUS negative. The plants which did prove GUS positive were checked for Agrobacterium contamination, to ensure no false positive results were obtained.

Table 5.2.2. The regeneration ability of explants inoculated with LBA4404:PBI121 and GUS assay results of regenerated plantlets.

	Tayberry		Sunberry		Loch Ness		8242E6		Sun Contrl.	
	LD	IS	LD	IS	LD	IS	LD	IS	LD	IS
No. expl.	100	100	100	100	100	100	100	100	100	100
No. died	62	54	60	54	58	54	69	62	39	35
No. callus	19	17	17	19	21	19	21	24	22	19
No. regen.	19	29	23	27	21	27	10	14	39	46
Plants	21	36	25	30	21	32	10	17	46	65
Plantlet Assay										
Died before assay										
	10	16	17	14	11	17	4	7	5	6
GUS +	4	7	3	8	4	7	3	4	0	0
GUS -	7	13	5	8	6	8	3	6	41	59

Slow growth of inoculated explants was again observed in this experiment with the inoculated regenerated

plantlets obtaining a height of 2 cm after a period of 22 weeks.

Experiment 5.2.3.

The inoculation of Ribes spp. with LBA4404:PBI121

Plant Genotypes: Ben More
Jet

Bacteria: LBA4404:PBI121

No. explants: 100 internodal segments / genotype

Media composition: Liquid NA
Solid NA

Hormone combination: 0.1 mg/l IBA and 2 mg/l BAP

Antibiotics: Carbenicillin

Experimental procedure: As experiment 5.1.10, using 400 mg/l carbenicillin as an antibiotic dip.

Results experiment 5.2.3.

These results given in table 5.2.3 on the following page, show a high proportion of explants regenerating which had not been observed with Rubus spp.

Also in proportion to the number of plantlets regenerating, a large number survived to be assayed. With the cv. Ben More a large proportion of regenerated plants were GUS positive and later confirmed by southern blot to be transformed.

Table 5.2.3. The regeneration ability of explants inoculated with LBA4404:PBI121 and GUS assay results of regenerated plantlets.

	Inoculated		Controls	
	Ben More	Jet	Ben More	Jet
No. explants	100	100	100	100
No. died	42	56	40	43
No. Regen.	50	39	60	57
Plantlets prod	70	54	98	102
Plantlet Assay				
No. plants assayed	65	49	89	94
No. GUS positive	38	24	0	0
No. GUS negative	27	25	89	94

Experiment 5.2.4.

The inoculation of Fragaria spp. with LBA4404:PBI121.

Plant Genotypes: Rapella

Rhapsody

No. explants: 50 leaf discs / genotype

Bacterial isolate: LBA4404:PBI121

Media: NA

Hormones: 0.2 mg/l BAP and 0.2 mg/l 2,4-D

Antibiotics: Carbenicillin at 400 mg/l

Experimental procedure: As exp. 5.1.10.

Results Experiment 5.2.4.

Table 5.2.4. The regeneration ability of explants inoculated with LBA4404:PBI121 and GUS assay results of regenerated plantlets.

	Inoculated		Control	
	Rapella	Rhapsody	Rapella	Rhapsody
No. explants	100	100	100	100
No. died	48	63	42	57
No. Regen.	17	14	30	19
No. Plantlets	22	16	34	23
Plantlet Assay				
No. plants assayed	16	9	25	17
No. GUS positive	6	3	0	0
No. GUS negative	10	6	25	17

These results are similar to the results for Rubus spp., with a large number of inoculated explants dying before assay. Around 35% of regenerated plants which survived to be assayed were transformed.

Experiment 5.2.5.

The inoculation of Vaccinium spp. with LBA4404:PBI121

Plant Genotypes: North Sky

North Country

Bacterial isolates: LBA4404:PBI121

No. explants: 40 leaf sections / genotype

Media: WPM

Hormones: DMAAP 3 mg/l.

Antibiotics: Carbenicillin at 400 mg/l

Experimental procedure: As exp 5.1.10.

Results experiment 5.2.5.

Table 5.2.5. The regeneration ability of explants inoculated with LBA4404:PBI121.

	Inoculated		Controls	
	North Sky	North Country	N.Sky	N.Country
No. explants.	40	40	40	40
No. died	34	32	23	22
No. callused	6	8	12	9
No. regenerated	0	0	5(13%)	9(23%)
No. plantlets	0	0	9	12

No regeneration was achieved from inoculated explants in this experiment. This experiment was repeated to determine if this failure was due to inoculation or variability in the regeneration process.

Experiment 5.2.6.

The inoculation of Vaccinium spp. with LBA4404:PBI121

Plant Genotypes: North Sky

North Country

Bacterial isolate: LBA4404:PBI121

No. explants: 100 leaf sections / genotype

Media: WPM

Hormones: DMAAP 3 mg/l.

Antibiotics: Carbenicillin at 400 mg/l

Experimental procedure: As exp 5.1.10

Results Experiment 5.2.6.

Table 5.2.6. The regeneration ability of explants inoculated with LBA4404:PBI121.

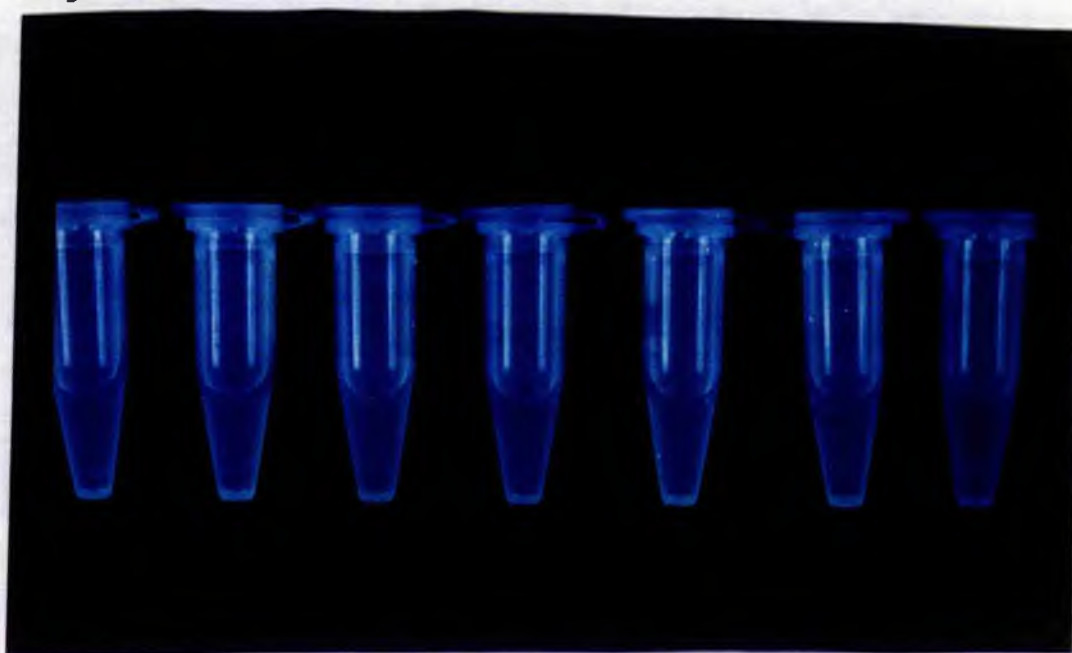
	Inoculated		Controls	
	N. Sky	N. Country	N. Sky	N. Country
No. explants:	200	200	200	200
No. died	148	158	82	84
No. callused	52	42	78	59
No. regenerated	0	0	40 (20%)	57 (29%)
No. plantlets	0	0	62	96

Again no regeneration was achieved from inoculated explants. The non-inoculated controls, however, did regenerate plantlets.

The following figures (5.4-5.5) clearly show the difference in assay results between transformed and non transformed plants. The blackcurrant cv. Ben More was used in the following assay. The transformed plants (5.5) show increased fluorescence over time due to the GUS enzyme breaking down the substrate to produce the product which fluoresces. With the non-inoculated plant (5.4), the GUS gene was not present, and therefore no enzyme was available to break down the substrate, consequently no fluorescent product was produced.

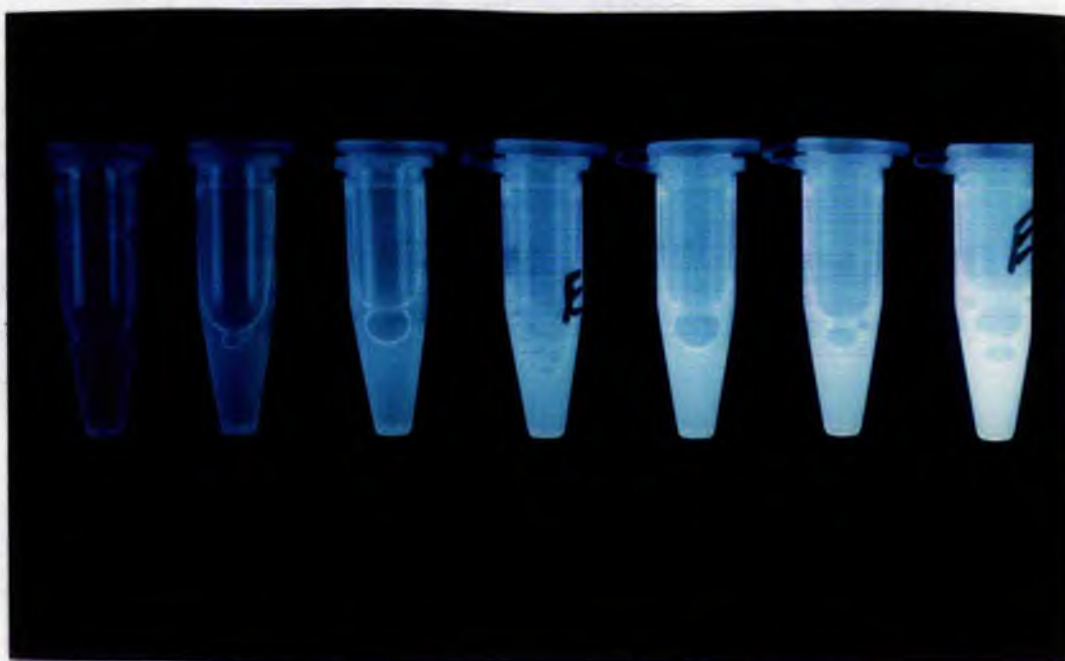
A GUS assay on a 10 ml overnight culture of LBA4404:PBI121 is also shown (5.6) where slight fluorescence can be seen over the whole time range of the tubes though again increasing with time.

Fig. 5.4



0 5 10 15 30 45 60

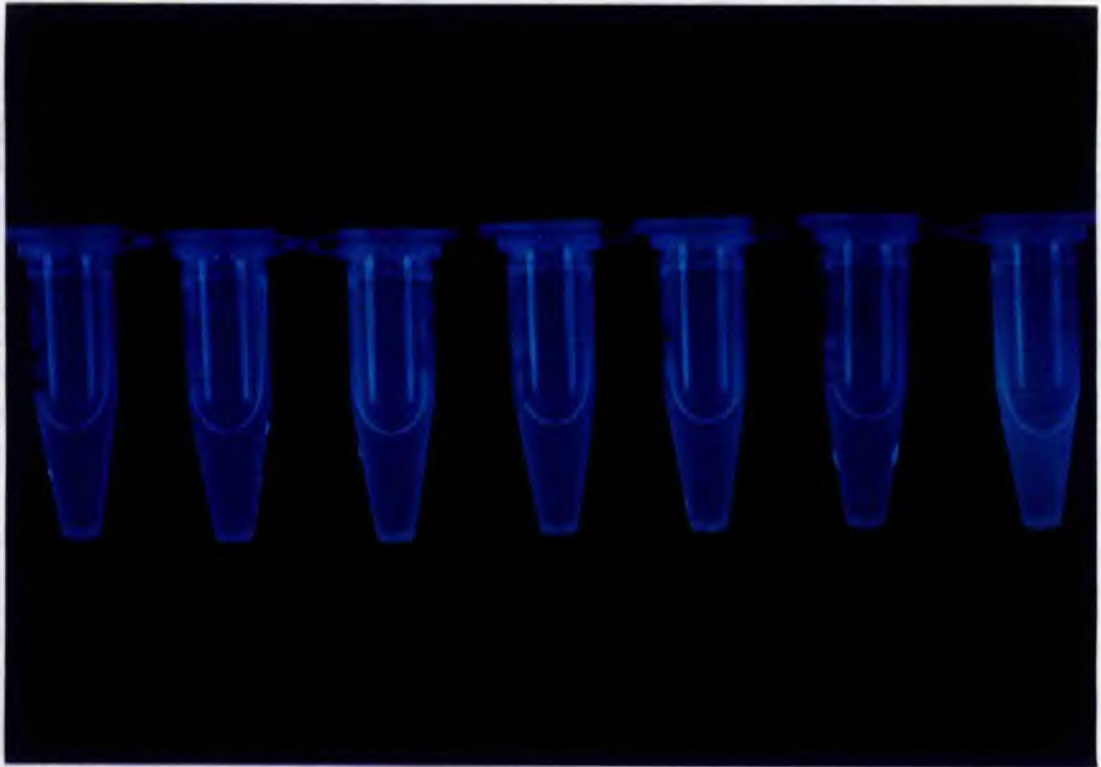
Fig. 5.5



0 5 10 15 30 45 60

GUS assay over time (0-60 mins) on a non-inoculated plantlet (5.4) and on a plantlet regenerated from explants inoculated with LBA4404:PBI121 (5.5).

Fig. 5.6



0 5 10 15 30 45 60

GUS assay on a 10 ml overnight culture of Agrobacterium isolate LBA4404 carrying the binary vector PBI121. A slight increase in fluorescence over time (0-60 mins) can be detected.

Experiment 5.2.7.

GUS assays of transformed plants at various times after transformation to determine if gene loss occurred.

This experiment involved carrying out GUS assays on a number of plants at various times after the plants had been first assayed.

Plant Genotypes: Tayberry

Sunberry

Ben More

Rapella

GUS Assay: As described previously.

Results Experiment 5.2.7.

GUS assays at various times after transformation.

In no case was a plant which had initially been found to be GUS positive, found at a later date found to be GUS negative. Also, no plants found initially to be GUS negative were GUS positive at a later date.

Experiment 5.2.8.

The amount of GUS expression in individually transformed plants.

The amount of GUS expression was measured in a range of plants to determine how variable the expression of a foreign gene might be.

Known standards of methylumbelliferone (MU) were prepared and 1uM MU was calibrated to equal 100 relative fluorescence. Non-inoculated control plants, which were assayed in the same way as inoculated plants, were used as blanks.

Plant Genotypes: Ben More

Rapella

Loch Ness

Experimental procedure: GUS assays were carried out as described in section 5.0. A total of 13 plants and 4 controls were examined.

Relative fluorescence to a known standard was measured.

Results Experiment 5.2.8.

The table on the following page illustrates GUS expression over time from 13 independently transformed plants. A variability in the levels of expression can be seen.

Table 5.2.8. uMoles MU produced over time from 13 individually transformed plants and 4 controls.

		TIME mins.				
		0	15	30	45	60
Genotype						
Ben More		0	0.16	0.33	0.64	0.90
Ben More		0	0.2	1.2	2.5	3.25
Ben More		0	0.03	0.45	1.26	1.90
Ben More		0	0.09	0.8	1.6	2.6
Ben More		0	0.01	0.4	0.9	1.3
Ben More		0	0.06	0.61	1.39	2.10
Rapella		0	0.03	0.25	0.46	0.78
Rapella		0	0.022	0.32	0.67	1.0
Sunberry		0	0.026	0.59	1.30	2.0
Sunberry		0	0.07	0.62	1.23	1.90
Sunberry		0	0.033	0.64	1.32	2.20
Loch Ness		0	0.015	0.16	0.32	0.455
Loch Ness		0	0.035	0.345	0.655	1.00
Ben More	C	0	0	0	0	0
Rapella	C	0	0	0	0	0
Sunberry	C	0	0	0	0	0
Loch Ness	C	0	0	0	0	0

The graph on the following page illustrates the variability in GUS expression.

Fig. 5.7

GUS assay over time from 13 individually transformed plants.

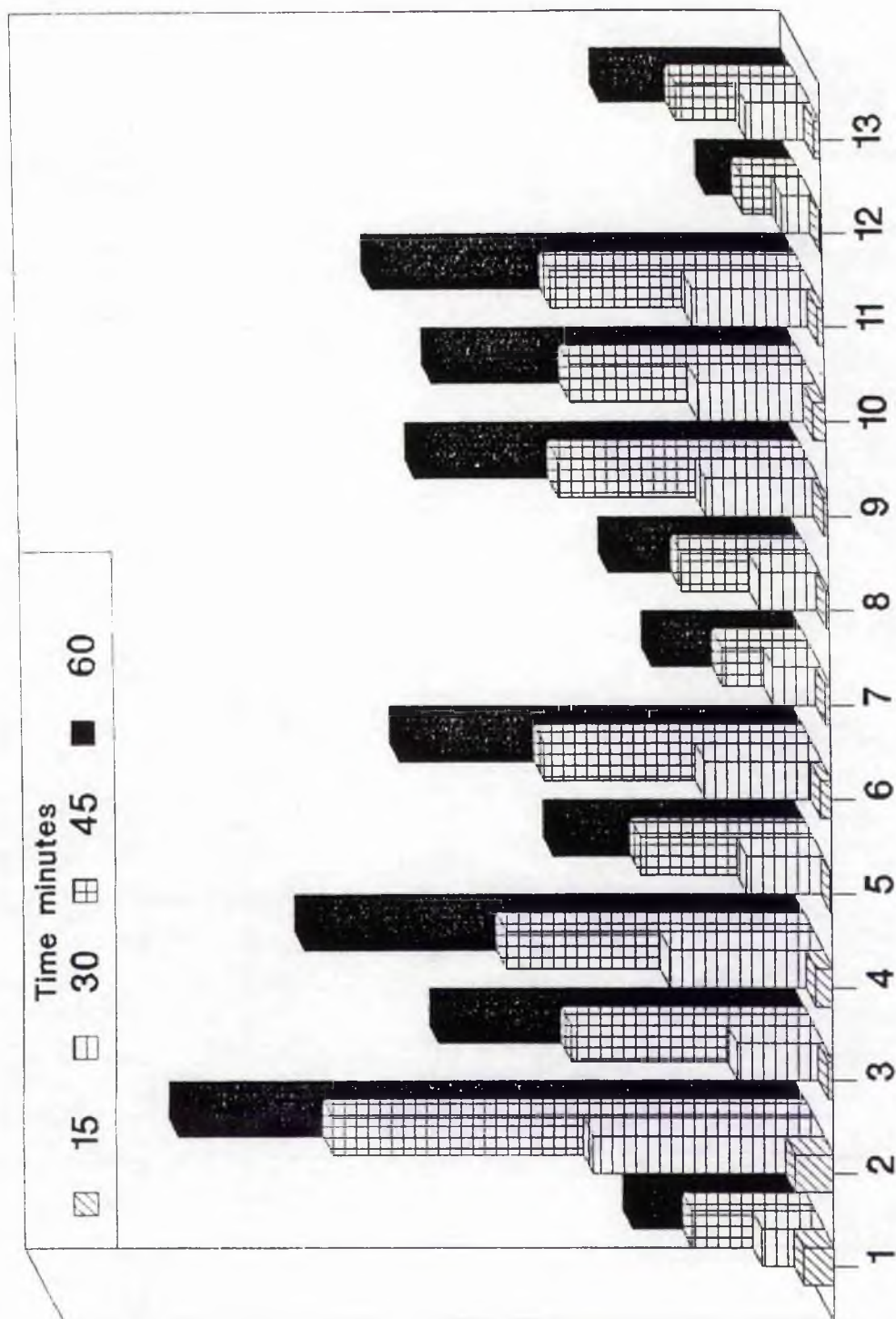
1-6: GUS assay on 6 regenerated plantlets of the blackcurrant cv. Ben More.

7-8: GUS assay on 2 regenerated plantlets of the strawberry cv. Rapella.

9-11: GUS assay on 3 regenerated plantlets of the hybrid berry Sunberry.

12-13: GUS assay on 2 regenerated plantlets of the blackberry cv. Loch Ness.

GUS activity over time from 13 individually transformed plantlets.



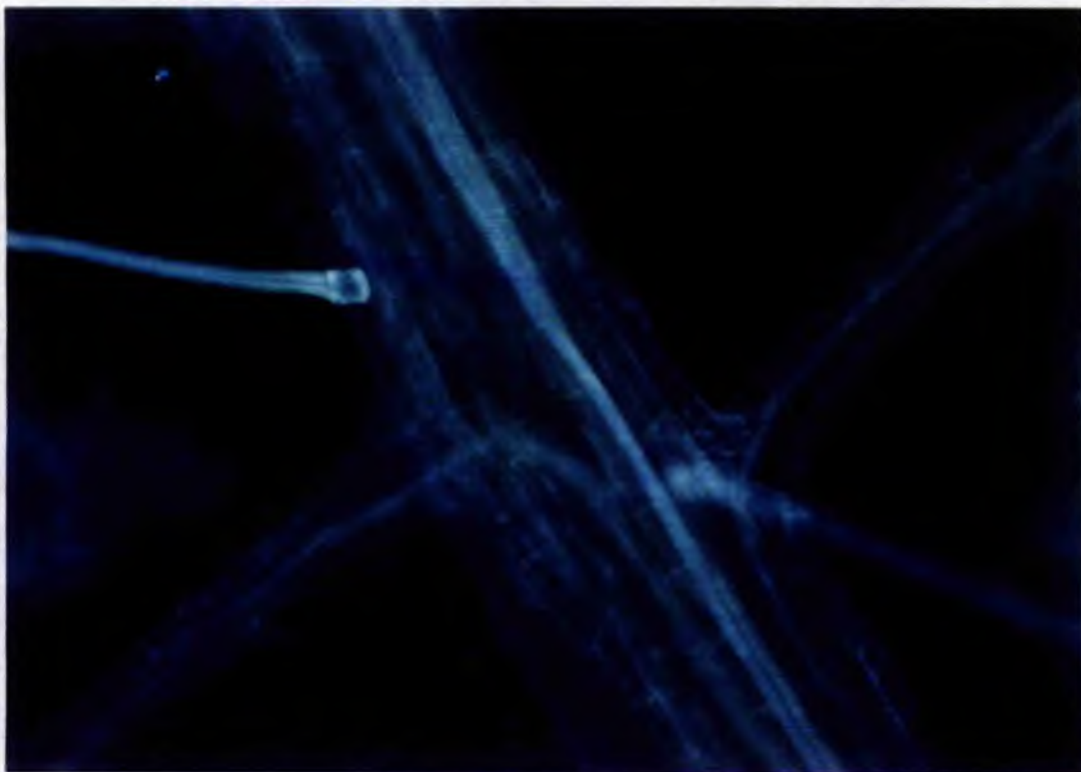
Experiment 5.2.9.

Histological staining of transformed leaf tissue.

Leaf material from transformed plants was fixed according to one of the procedures described in section 1 and stained with X-Gluc.

The following figure (5.8) is typical of the result obtained on transformed plant material. The leaf tissue was cleared before examination and the blue regions indicate areas of gene expression.

Fig. 5.8

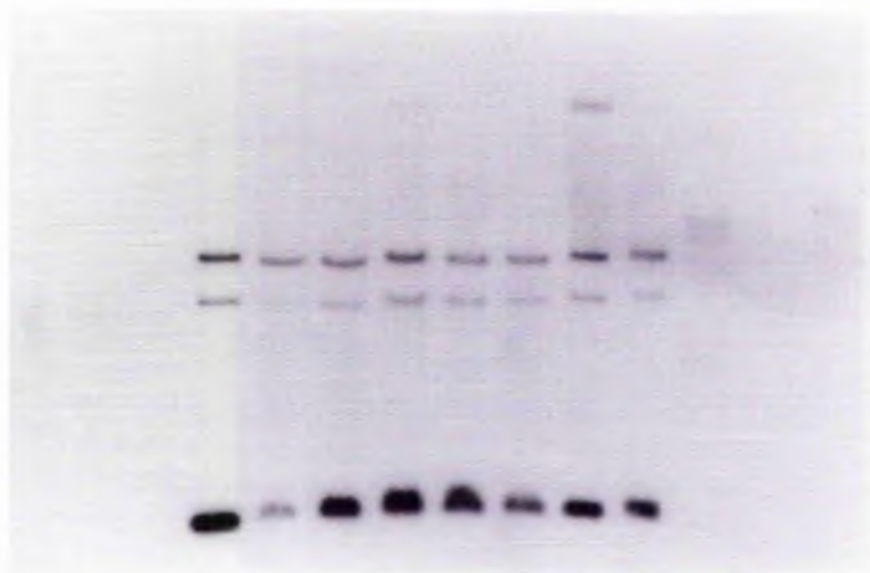


X-GLUC staining of cleared leaf tissue from a plantlet transformed with the beta-glucuronidase gene. The mid-vein can clearly be seen. A blue precipitate indicates gene expression.

Only very limited Southern hybridisation assays have been carried out on the transformed plant material. The figure on the following page (5.9) is showing bands due to hybridisation of the radioactive probe with genomic DNA. In total twelve lanes were run, the lanes containing the control plants are very faint and the last lane containing the molecular weight markers is very vague.

From left to right the visible lanes contain two samples from the cv. Rapella, three samples from the cv. Ben More and 3 samples from the cv. Tayberry.

Fig 5.9



A B C D E F G H I J K L

Southern blot of DNA extracted from plants regenerated from explants inoculated with LBA4404:PBI121.

A control Tayberry
B control Rapella
C control Ben More
D Rapella
E Rapella
F Ben More
G Ben More
H Ben More
I Tayberry
J Tayberry
K Tayberry
L Molecular weight markers

Discussion.

This chapter dealt with experiments leading to the transformation of Rubus, Ribes and Fragaria spp. and the attempted transformation of Vaccinium spp.

Initially the binary vector Bin 19 was used in an attempt to transfer the NPT II gene into plant material. Before this was used in inoculation experiments, two preliminary investigations were carried out. The first examined the effect of the antibiotic kanamycin on non transformed plants, and the second examined the effect of inoculation with Agrobacterium (not containing a binary vector) on explant regeneration.

The antibiotic kanamycin was shown to have a whitening effect on plant material, and depending on the concentrations used this effect could be achieved on all plants tested. This demonstrated that the NPT II gene, if integrated and expressed in the plant material, would be of use as a selectable marker gene. If kanamycin had been shown to have little or no effect on the plant material then this gene would not have been of great use.

The second experiment demonstrated a significant reduction in internodal segment regeneration when inoculated with Ar9402, and a lesser though not significant reduction in leaf disc regeneration. Regeneration of leaf discs and internodal segments inoculated with LBA4404 was also reduced though to a much lesser extent. This greater reduction in

regeneration due to Ar9402 can be explained by the presence of hormone genes on the Ar9402 Ti-plasmid, not found on the disarmed Ti-plasmid of the LBA4404 isolate. Regeneration from control explants was also reduced, and this must have been caused by the process of inoculation (though no Agrobacterium was present), incubation, and antibiotic dips.

The initial transformation experiment (5.1.3) failed due to 100 mg/l of carbenicillin in the regeneration medium failing to control Agrobacterium contamination. This experiment was repeated (exp.5.1.4) using a weaker inoculum with which contamination was not as much of a problem; however, the explant material began to die after 10 days and by 21 and 28 days respectively, all explants were dead.

At this point I decided to examine the effect that antibiotic medium was having on explant regeneration and survival. Experiments (5.1.5-5.1.7) examined the effects of kanamycin, carbenicillin and cefotaxime on explant regeneration. Kanamycin at a concentration of 25 mg/l or more completely inhibited plantlet regeneration from both leaf discs and internodal segments. Carbenicillin greatly reduced regeneration at 50 mg/l to approximately 8% for leaf discs and 16% for internodal segments, and at 100 mg/l to 1.6% for leaf discs and 6.6% for internodal segments. Cefotaxime at 50 mg/l also completely inhibited explant regeneration.

These experiments demonstrated a devastating effect on explant regeneration due to the presence of antibiotics in the medium.

In the experiments which followed (exp.5.1.8-5.1.10) the amount of time the explants were in contact with antibiotics was reduced. However in exp.5.1.8 it was established that as little as 3 days on carbenicillin medium and 2 days on kanamycin selection medium was enough to completely suppress leaf disc regeneration and with internodal segments only 1 of the 30 segments regenerated. In exp.5.1.9, explants were placed onto carbenicillin or cefotaxime medium for 4 days after which time they were placed onto medium without antibiotics. Only after regeneration had started to occur were explants placed onto the selection medium. Cefotaxime again almost completely inhibited explant regeneration. With carbenicillin, regeneration occurred to a small extent. Of the 27 plantlets which regenerated from 21 explants (out of a total of 240 explants on carbenicillin), 9 died soon after regeneration and the other 18 were placed onto kanamycin-containing medium. Of the 18 plants on selection medium, 7 remained green suggesting possible transformants. As expected, none of the 24 control plantlets which regenerated (from 20 explants out of a total of 120 on carbenicillin) remained green on selection medium.

Contamination became a problem once the explants were removed from the carbenicillin medium and antibiotic

dips were required to prevent the explants becoming overrun with Agrobacterium.

Experiment 5.1.10 utilised antibiotic dips at 2 different concentrations (200 mg/l and 400 mg/l), rather than antibiotic-containing media to remove Agrobacterium contamination. Carbenicillin at 200 mg/l failed to control Agrobacterium contamination which completely overran the explants. The number of explants regenerating following the 400 mg/l dip was slightly more than double that in the previous experiment, 51/240 (21%) compared with 22/240 (12%) and the number of control plants regenerating also increased with 93/240 (39%) regenerating compared with 20/120 (17%). Of the inoculated plantlets which regenerated, 19/53 (36%) remained green on kanamycin medium whereas 100% of control plants (93/93) whitened.

To confirm transformation, Southern hybridisation and dot blot assays were carried out on the putative transformants, i.e. those plants which remained green on kanamycin.

Because both of these procedures for identifying transformants proved time consuming even on a small number of plants and controls, the Beta-glucuronidase enzyme which has a very reliable and less time-consuming assay was used as the marker in all further experiments. Experiment 5.2.1 involved inoculating explants with LBA4404 carrying the binary vector PBI121, containing the coding sequence of the GUS enzyme. As in exp.5.1.10, antibiotic dips were used in place of antibiotic media.

Of the plants inoculated with Ar9402 only 6/240 (2.5%) explants regenerated producing 13 shoots of which 6 died; 3 were GUS positive and 4 GUS negative. With LBA4404, 54/240 (or 23%) explants regenerated producing 71 shoots of which 22 died before assay; 19 of these were GUS positive and 30 GUS negative. Of the controls 99/240 explants regenerated producing 110 plantlets of which 15 died before assay; the remaining 95 were GUS negative. Experiment 5.2.1 was repeated (exp.5.2.2) using LBA4404:PBI only, to examine how consistent the results were.

In experiment 5.2.2 out of a total of 800 inoculated explants 170 (or 21%) regenerated a total of 192 shoots. Of these plantlets 96 died before assay; 40 of these were GUS positive and 56 were GUS negative. Of the number of plantlets which regenerated (24%), and subsequently survived to be assayed (30%), 42% were transformed, this compares favourably with the previous experiment where 39% of those plants that survived were GUS positive (23% regenerating). Control plants in this experiment regenerated to a greater extent with 43% of explants producing shoots.

Experiment 5.2.3 attempted to transform 2 blackcurrant genotypes with LBA4404:PBI 121. A high proportion of explants regenerated (45%) compared with the Rubus results. Of the plants which survived (generally most of the regenerated plantlets) to be assayed, a high proportion of them were transformed (54%).

In the transformation of strawberries, similar results to the Rubus experiments were obtained with a low number of regenerants (16% of inoculated explants regenerating) (25% controls regenerated). Of the plants which survived to be assayed 36% of them were transformed.

Experiments involving the inoculation of Vaccinium spp. failed to produce any regenerants, and regeneration from non-inoculated controls was also greatly reduced (18% exp.5.2.5 and 24% exp.5.2.6). Both the Agrobacterium and the antibiotic dips must have had a severe effect on the blueberry leaves.

Slow growth of a number of plantlets regenerating from inoculated explants was observed throughout all the transformation experiments. Too small a number of plantlets had regenerated, however, to enable significant differences to be observed in plant growth between explants shown to be transformed and those shown to be non-transformed. Experiments are now under way to determine if slow growth is a feature of the inoculation process, or if it is due to gene insertion in soft fruit. If slow growth is found to occur at a significantly greater level in inoculated plants shown at a later date to be transformed than in inoculated plants shown not to be transformed, then this would provide an initial screen of regenerants in future experiments.

Two further experiments examined the possible loss in expression of the inserted gene over time, and also

variation in gene expression over transformed plants. It was established that none of the transformed plants tested had exhibited a loss in gene expression after a period of 1 year. However, variation in enzyme activity was found between individually transformed plants.

A final experiment involved staining leaf tissue with X-Gluc, a substrate for histochemical localisation of beta-glucuronidase activity in tissues. This substrate forms a blue precipitate at the site of enzyme activity. Blue areas can be seen along the mid-vein and elsewhere in the leaf showing the presence of the GUS enzyme in leaf tissue.

These initial transformation experiments in soft fruit have shown that for Rubus, Ribes and Fragaria spp. it is now possible to insert genes using Agrobacterium as a vector. Gene transfer to the progeny and gene loss have still to be determined; however, due to the slow growth of transformed plantlets and the growth cycle of soft fruit in general, examination of those features is still some time away.

Although initial transformation experiments have still not reached completion, it was decided to begin experiments using potentially useful genes rather than marker genes.

The next chapter describes the genes available now for transfer into soft fruit and the problems they may overcome.

Chapter 6

**Use of the developed transformation system in soft
fruit.**

Use of the developed transformation system in soft fruit.

Introduction

This chapter, which is the final chapter to contain results of experimental work, is concerned with research recently initiated to overcome some of the problems associated with soft fruit, using the developed transformation system. Due to the length of time required to complete the life cycle in soft fruit, it has not been possible to study the inheritance of inserted marker genes (Chpt.5), i.e. their transfer to progeny. However this chapter reports research now underway that is aimed at inserting potentially useful genes into soft fruit. It is hoped that Mendelian transmission of these genes to the progeny will occur, and the loss of A. tumefaciens-inserted genes will be in line with natural gene loss.

In considering plant improvement, there are a number of goals to which the new genetic engineering techniques can be applied. These include extending the capacity to fix atmospheric nitrogen to cereals and other crops, conferring tolerance to drought, salinity, cold and flooding, alteration of the photosynthetic mechanism, regulation of gene expression and the development of improved disease and pest resistance.

A major objective for soft fruit crops (and the majority of other crops), is the production of plants with durable pest and disease resistance. Insect pests are responsible for heavy crop losses and their control requires high expenditure on insecticides throughout the world. Approximately 37% of all crops produced world wide are lost to pests (Gatehouse and Hilder 1988).

Due to the variety of pests which attack soft fruit plants, a large number of sprays are applied each year to control crop damage. The majority of these sprays are applied at a stage when the fruit is developing and therefore, as well as posing a potential hazard to the environment, they are also a potential hazard to the consumer. Increasingly, the use of chemicals in the environment is becoming unacceptable and alternative methods of protection are required. The only real alternative to chemicals has been inherent plant resistance, manipulated until recently by plant breeding procedures.

With the availability of recently developed plant vector techniques, the insertion of resistance genes from novel sources is now an alternative to chemical control and plant breeding. These techniques overcome the limitations of plant breeding, and the use of inserted resistance genes has advantages over chemical control. Aside from the great financial saving of not using pesticides, genetic control removes the need to spray seasonally at critical insect stages and protection is not dependent on the weather or the

effectiveness of spray coverage. On an environmental level, only insects eating the crops will be affected and the consequential reduction in the use of chemicals also lessens environmental pollution and removes danger to the consumer from residues.

At present the availability of resistance genes is limited; however, with the increasing technology for gene isolation, coupled with identification of potentially useful genes, it should only be a matter of time before this restraint on the usefulness of the system is overcome.

Immediate possibilities exist in soft fruit for pest and virus control by the genetic engineering of resistance genes.

Examples of inserted characteristics of agricultural importance include herbicide resistance (Comai et al. 1985, Shah et al. 1986) (though the importance of herbicide resistance genes has been the subject of much debate (Chpt.7)), virus resistance (Baulcombe et al. 1986, Abel et al. 1986), and insect resistance by the Bacillus thuringiensis endotoxin genes (Vaech et al. 1987, Fischhoff et al. 1987) and the cowpea protease trypsin inhibitor (CpTi) gene (Hilder et al. 1987). The CpTi gene could potentially be very useful in soft fruit.

Serine protease inhibitors are abundant in seeds and storage organs, such as tubers of a wide range of dicotyledonous and monocotyledonous plants. Due to their function as natural protective compounds against

herbivorous insects, interest has been shown in the transfer of the gene into crop plants by Agrobacterium. The Bowman-Birk trypsin inhibitors from the cowpea (Vigna unguiculata L. (Walp)) have been implicated in field resistance to its major insect pest and shown to be anti-metabolic to a wide range of herbivorous species (Gatehouse and Hilder 1988).

Research leading to the isolation of the cowpea protease trypsin inhibitor originated at the International Institute for Tropical Agriculture (IITA) in Nigeria. The cowpea Vigna unguiculata, is a staple food source in West Africa and South America. Extensive losses of the cowpea occur during storage due to feeding by the bruchid beetle, Collosobruchus maculatus F. Researchers at IITA identified one line out of fifteen thousand showing dramatically increased resistance to the beetle compared with other genotypes (Singh, 1978).

Researchers at the University of Durham then identified the basis of resistance in that particular line. The presence of secondary anti-metabolic compounds was looked for and only inhibitory activity against the proteolytic enzyme trypsin and to a lesser extent chymotrypsin were detected. The resistant variety contained at least twice the amount of trypsin inhibitor than that of any other varieties.

Once the anti-metabolic effect of the inhibitor in feeding trials had been demonstrated, the corresponding gene was isolated, cloned (Hilder et al. 1987), inserted into a binary vector and used to transform tobacco

plants (Nicotiana tabacum cv. Samsun N.N.).

Transformants were then tested against a range of pests which normally attack tobacco plants along with non-transformed controls. Significant differences in the results of larvae feeding on the controls and transformants were obtained (Gatehouse and Hilder 1988).

The cowpea protease trypsin inhibitor gene is of interest in soft fruit, where a number of insect pests can cause serious damage and need to be controlled by insecticides or acaricides. The following section gives brief descriptions of a number of pests of soft fruit which may be eliminated or their destructive effect significantly reduced by insertion of the CpTi.

Rubus species.

The most important pest of raspberries is probably the raspberry beetle. This beetle (Byturus tomentosus Degeer) is found throughout the U.K. in commercial and wild red raspberry, hybrid berry and blackberry plantations. The adult beetles feed on buds and leaves of the primocanes (Taylor 1971, Taylor and Gordon 1977). As the fruit starts to ripen, the larvae tunnel into the softening receptacles and feed on the undersides of the maturing drupelets. Aside from damage, actual larval contamination of the fruit renders it completely unacceptable to the consumer.

At present damage and larval contamination are controlled only by prophylactic sprays of insecticides which are applied between the 80% petal fall stage and

the first pink fruit stage (Jennings 1988) and this spraying at such a late stage is becoming increasingly less acceptable.

Another major, though not annually occurring, pest of raspberries is the raspberry cane midge (Resseliella theobaldi Barnes). This is distributed in most European raspberry growing areas where it emerges during warm years (McNicol et al. 1983). Larvae feed under the bark and fungi invade the feeding sites giving rise to the disease complex known as midge blight (Pitcher and Webb 1952). Control is again by the use of chemical sprays which are applied to control the first generation and thus minimise the second generation which cannot be sprayed as its emergence coincides with fruit harvest (Gordon et al. 1990).

The third major group of pests of raspberries are the raspberry aphids (Hemiptera : Aphididae). Aphids transmit several important viruses including Rubus yellow net virus, raspberry leaf mottle, raspberry leaf spot virus and black raspberry necrosis virus. Aphids are not chewing insects and will not be controlled by the CpTi; however, as described in the section on virus control which follows, there are other possibilities.

A number of minor pests exist which can be severe in certain areas or in some years. The clay-coloured weevil (Otiorhynchus singularis L.) is not a widespread problem in the U.K., although local infestations occur. The weevil is frequently a serious raspberry pest,

feeding on the petioles of leaves on the fruiting laterals causing wilting and damage to the developing flower bud, and a strawberry pest, eating the roots. Before the introduction of DDT in the early 1950s, the clay-coloured weevil was regarded as one of the most important pests of raspberries in Scotland, and there are signs that its importance may again be increasing (Gordon 1990). Control can be achieved adequately by applying organophosphorous insecticides during the night when the weevils are actively feeding on the raspberry foliage (Gordon and Woodford 1986).

Another pest which declined with the introduction of DDT but again may be on the increase, is the raspberry moth (Lampronia rubiella Bjerkander). The raspberry moth is found throughout Britain, and can be a serious pest in Scotland. Caterpillars emerge in early April and bore into the bursting buds which subsequently fail to develop. Spray programmes for raspberry beetle give adequate control.

The double dart moth (Graphiphora augur Fabricius.) is a pest which appears irregularly, disappearing for a large number of years. In Scotland it has been a problem for the last two years though it had not been seen for the previous 17 years (Gordon and Lawson 1989). The moth has a patchy but wide distribution and was first discovered feeding on red raspberry in eastern Scotland in the early 1980s (Gordon et al. 1988). The moth feeds on developing buds and leaves, low numbers of which can

cause severe damage. Control can again be achieved by a nocturnal application of organophosphorous insecticides.

Other minor pests exist for raspberries including the tortrix moth and the raspberry crown borer. The tortrix moth can also be a problem in strawberries where spraying occurs before fruiting. The raspberry crown borer is a serious pest in North America but not in Europe.

Fragaria species.

Pests of importance in strawberry plantations include red spider mites (Panonychus ulmi Koch) and the two-spotted spider mite (Tetranychus urticae Koch) which can cause serious damage to strawberry plants under conditions favourable for mite infestation. Spider mites cause scarring and bronzing of leaf tissue and reduced plant vigour. Root weevils can also cause serious damage to strawberry plants. Adult root weevils feed on the leaves of strawberry plants but the major damage results from larval feeding on the roots (Barritt and Shanks 1980).

Vaccinium species.

Domesticated entirely in the twentieth century, blueberry culture in Britain at this time is fairly free of insect pests, the only control measure necessary at the moment is an occasional spray against the tortrix moth (Cormack 1979).

With increasing acreages, a number of pests may become important, among these are the blueberry bud mite (Acalitus vaccinii Keifer), a predator of unopened buds. Heavy infestations kill the buds and lead to reduced yield. Control is achieved with a post harvest application of chemicals.

Flower buds may be damaged by feeding of the cutworm caterpillars (Rhynchagrotis species) and measuring worms (Eupithecia species). These caterpillars chew large irregular holes in the blueberry bud causing it to turn brown and die.

Cranberry weevil (Anthonomus musculus Say) can be a serious pest in the northeastern United States. It emerges in early spring and punctures the slightly expanded flower and leaf buds. Punctured flower buds often fail to open.

The blueberry maggot (Rhagoletis mendax Curran) is the major fruit-destroying insect in northern United States. The adult fly inserts its eggs just under the skin of the ripening blueberry and up to 3 insecticide treatments are necessary to control adults (Vincent and Lareau 1989).

The plum curculio (Conotrachelus nenuphar Herbst) is another important economic pest that attacks blueberry fruit. The adult weevil lays a single egg in a crescent shaped depression that it makes in the centre of the immature green fruit. Up to 140 eggs may be laid by a single female. The larva bores into the centre of the

fruit and eats its way through the contents of the berry which then prematurely drops to the ground.

The adult moths of the cranberry fruitworm lay their eggs within the green calyx cup of green berries. A larva enters the berry at the junction of the stem and the fruit where it remains concealed until the contents of the fruit has been consumed, whereupon it tunnels into another fruit. Infested fruit will turn prematurely blue and shrivel.

Of the foliage feeding insects that attack the blueberry the most serious is the sharpnosed leafhopper (Scaphytopius magdalensis Provancher). This leafhopper carries the mycoplasma that causes stunt disease. The insects themselves doing little damage to the plant.

Four species of leafminer attack the blueberry of which Graciliaria vacciniella Ely is possibly the most common.

The blueberry crown girdler (Cryptorhynchus obliquus Say) is the larva of a weevil that attacks only the crown of the blueberry bush and is capable of completely girdling and killing the crown.

Descriptions and information on the major blueberry pests are given by Eck (1988).

Ribes species.

Spraying is required against a number of pests including the blackcurrant gall mite (Cecidophyopsis ribis West). This sucks sap from the interior of the bud

and gives rise to the characteristic swellings known to growers as 'big bud'. These infected buds fail to produce leaves or flowers during spring. Control depends on contact action of an acaricide.

Feeding by caterpillars of the blackcurrant sawfly (Nematus olfaciens Benson) causes damage to the foliage in the centre of the bush during spring and summer. In late spring the adult fly lays small white eggs along the undersides of leaves in the centre of the bush. The continued feeding of the caterpillar results in the leaf tissue being completely eaten away. Control is achieved by high volume application of chemicals.

The leaf curling midge (Dasyneura tetensii Rubs) badly stunts the growth of young bushes. Distortion of the leaves at the shoot tip is clearly visible. Leaf margins curl inward and the leaf becomes permanently distorted. The adult midge emerges from pupation towards the end of the flowering period and lays eggs close to the mid-rib of a young leaf which curls and protects larvae. Control is difficult to achieve using contact insecticide, because the eggs are laid over a lengthy period and protection occurs due to leaf curling.

The common green capsid (Lygocorus pabulinus L.) can also stunt the growth of young plantations by feeding on the leaves causing serious damage. Leaves near the tips of young shoots take on a yellow appearance. Nymphs hatch in spring from eggs laid on the shoots late in the preceding summer.

Red spider mites (Tetranychus urticae Koch) feed on blackcurrant bushes in the field. In winter the adult lives concealed in straw or other debris at soil level. The mite feeds by sucking sap from the underside of leaves producing the characteristic bronzing on each side of the midrib. In spring mites leave their overwintering shelter and ascend the bushes to feed on young foliage. If a large population exists initially then mites rapidly spread. (Anon. 1973).

The above description of important pests which cause significant loss and require chemical sprays illustrates the importance of pest resistant cultivars.

In addition to pests, a number of viruses are important in raspberry plantations and again virus resistance is an important aspect of the breeding programme.

The most effective method of preventing plant virus infection is by the use of resistant varieties. However there is often a lack of resistance within the crop species making plant breeding for resistance a difficult objective to achieve. Other methods of control involve elimination of the vector, generally by chemical control or genetic resistance. Another approach to the control of virus infection is the phenomenon of cross-protection.

The process of cross-protection involves inoculating plants with mild strains of a virus to prevent the development of severe symptoms when challenged with a

more virulent strain of the same virus. Cross-protection has successfully been used for the control of a number of plant viruses including papaya ringspot virus (Yeh and Gonsalves 1984) tobacco mosaic virus (Rast 1972 and Fletcher 1978) and citrus tristeza virus (Muller and Costa 1977). This procedure does carry the risk of the mild strain becoming more virulent or acting synergistically with other viruses. Studies on the molecular basis of cross-protection have indicated that the coat protein of the protecting virus has an important role in systemic cross-protection. Sherwood and Fulton (1982), studying tobacco mosaic virus (TMV) suggested that the protection results from an inhibition of uncoating of the virus, preventing the release of infectious RNA. In their studies they found that leaf areas containing high concentrations of virus in tobacco plants infected with a strain of TMV causing mosaic symptoms were not susceptible to infection by virions of a TMV strain causing necrotic lesions, whereas these areas were susceptible to infection by unencapsidated TMV RNA of the necrotic strain. Similar results were obtained by Dodds et al. (1985) on cross protection studies of tomato plants infected with cucumber mosaic virus.

The development of plant genetic engineering techniques has allowed individual viral genes to be inserted into plants to determine their involvement in cross-protection.

A number of researchers have found plants expressing the coat protein gene of a virus to be slower at symptom development than non-expressing plants. Abel et al. (1986) found that between 10 and 60% of their tobacco plants expressing tobacco mosaic virus coat protein never developed symptoms. Similar results were obtained by Loesch-Fries et al. (1987) from tobacco plants expressing the alfalfa mosaic virus coat protein gene.

An alternative strategy based on the use of virus or virus related sequences in plant protection involves viral satellites (Schneider 1969 and 1971). Satellites are small RNA molecules which biologically resemble a parasite being encapsidated with and dependent on, the virus genomic RNAs for replication. In many cases satellites have been found to either enhance or suppress the virus symptoms.

Murant et al. (1973) found a satellite of tomato black ring virus to be responsible for decreased or abolished infection by the viral RNAs. Kaper and Tousignant (1977) identified a satellite of cucumber mosaic virus, and found an increase in the amount of satellite correlated with a decrease in symptoms of the plants.

In other cases satellites have been found to increase disease severity. Kaper and Waterworth (1977) demonstrated in tomatoes that the presence of cucumber mosaic virus associated RNA 5 (CARNA 5) in inoculums of genomic RNAs, lead to the subsequent incidence of severe necrosis in tomato plants. On other crops (e.g.

sweetcorn and tabasco pepper), however, CARNA 5 had the opposite effect of reducing disease symptoms (Waterworth et al. 1979).

Again, due to developments in plant genetic engineering techniques, virus satellite sequences have been incorporated into plant vectors and inserted into plants to examine effects on virus infection.

Harrison et al. (1987) transformed tobacco plants with a DNA copy of satellite RNA of cucumber mosaic virus (CMV). They found that tobacco plants with a DNA copy of this satellite produce large amounts of the satellite RNA on infection with a satellite free inoculum of CMV. CMV replication was greatly decreased and symptom development largely suppressed in these transgenic plants. Satellite RNA synthesis is also induced by tomato aspermy virus (TAV) which is closely related to CMV and causes symptom suppression. This time, however, there is little decrease in TAV genome synthesis.

Gerlach et al. (1987) reported that transgenic tobacco plants expressing the satellite sequence of tobacco ringspot virus (TobRV) show phenotypic resistance when infected with TobRV.

Possibilities for the insertion of viral sequences into soft fruit are available which should combat arabis mosaic virus, one of a number of viruses causing serious disease in soft fruit.

Arabis mosaic virus (AMV), transmitted by the nematode Xiphinema species, is a major problem in

southern Britain. Several raspberry cultivars are sensitive to infection with AMV and it is a major problem in the commonly grown cultivars Admiral and Glen Clova. In addition to raspberry, AMV has been found in wild blackberry and also in strawberry. Symptoms usually do not develop until after the first year of infection. Symptoms typically consist of yellow flecks, mosaics or ringspots in expanding leaves and/or vein yellowing in older leaves. Plants are usually stunted, produce small berries and decline in vigour each year with great reductions in yield occurring after 3 years (Jones 1986).

Another important virus of Rubus spp. is raspberry bushy dwarf virus (RBDV), transmitted by pollen. RBDV occurs world wide where raspberry is grown, causing the raspberry yellows disease (Cadman 1965), symptoms of which occur in late spring as yellow vein netting of the lower leaves and/or general leaf chlorosis (Cadman 1952). In chapter 7 (on prospects of the developed system), research initiated into overcoming this disease will be briefly discussed.

Other viruses include the aphid-bourne raspberry leaf curl virus, raspberry vein chlorosis virus, Rubus yellow net virus, black raspberry necrosis virus and raspberry leaf spot virus. Among the nematode-borne viruses are AMV, described briefly above, and raspberry ringspot virus. Pollen borne viruses include RBDV already mentioned, apple mosaic virus and tobacco streak virus.

Brief descriptions of viruses in Rubus crops are given by Jones 1986.

In strawberry, arabis mosaic virus is important as are raspberry ringspot, tomato blackring and strawberry latent ringspots.

Virus diseases which are important in blueberries include blueberry stunt, the first virus disease known in cultivated blueberries, with characteristic symptoms of a yellowing of the leaf along the margins and between the lateral veins. Chlorotic areas turn brilliant red in late summer. Diseased plants live for many years but have reduced vigour. Red ringspot disease causes the characteristic red spots and rings. Others diseases include blueberry necrotic ringspot, witches broom virus and blueberry scorch, symptoms of which were first observed in 1980 and appear to be caused by two or possibly three viruses (McDonald et al. 1989).

A number of viruses attack blackcurrants, including reversion virus spread by gall mites is recognised by changes which occur in flower and leaf characters. Minor viruses include cucumber mosaic virus, blackcurrant infectious variegation virus, blackcurrant yellows virus and gooseberry vein banding virus.

In the following section I describe experiments carried out in an attempt to insert the CpTi gene, which offers the possibility of insect resistant plants, and the coat protein gene or a DNA copy of satellite RNA into Rubus and Fragaria spp. for control of AMV.

Materials and Methods.

Bacterial isolate.

LBA4404, a disarmed A. tumefaciens isolate described in chapter 5, was maintained on Luria-Bertani (LB) medium containing 50 mg/l of the antibiotic rifamycin at 4°C.

Binary Vectors.

The binary vectors were maintained in E. coli strain HB101 on LB medium with 50 mg/l of the antibiotic kanamycin.

All binary vectors used were derivatives of Bin 19, described in chapter 5, with a site containing the CaMv 35s promoter and nos terminator, between which the coding sequence of the CpTi, AMV satellite or AMV coat protein genes were inserted.

pROK/CpTi+5

This construct contains the coding region of the cowpea protease trypsin inhibitor gene.

pROK/CpTi+5 is on licence from Agricultural Genetics Company (AGC).

pROK A3/3

Here the DNA copy of the satellite sequence of Arabis Mosaic Virus (AMV) was inserted.

pROK CP+

In this vector the DNA sequence of the coat protein gene of AMV was present.

pROK A3/3 and pROK CP+ were kindly supplied by Prof. I. Cooper. Oxford.

Mobilisation of the binary vectors into LBA4404.

The binary vector required for inoculation was mobilised into LBA4404 by the triparental cross procedure described in chapter 5.

Preparation of the inoculum.

The inoculum was prepared by growing up 10 ml overnight cultures and resuspending them individually in 60 mls of liquid NA.

Plant genotypes.

Rubus spp.

Sunberry

Glen clova

Glen Moy

Fragaria spp.

selection 71WC64

Preparation of explant material.

Explants were prepared as described in chapter 3 and cultured on the appropriate regeneration media described in chapters 3 and 4.

The inoculation of explant material with LBA4404 containing a binary vector.

Inoculation was carried out in a 9 cm diameter petri dish into which 30 mls of the inoculum (prepared as described above) was added. After 20 mins, explants were removed from inoculum and incubated for 1 day on filter paper discs on solid NA medium.

Agrobacterium contamination was eliminated after incubation, by repeated antibiotic dips followed by dipping into sterile distilled water and placing onto fresh media.

Regeneration of inoculated explants.

After 1 day incubation on filter paper the explants were dipped into 400 mg/l carbenicillin, then sterile distilled water and placed onto regeneration medium. Only when regeneration had occurred were explants placed onto kanamycin selection medium.

Methods of identifying transformants.

- 1) Kanamycin selection medium.
- 2) Trypsin assay.
- 3) Southern blots.
- 4) Comparison of insect pest feeding trials on transformed and non-transformed leaf tissue.

5) Graft inoculation with virus-infected material.

1) Kanamycin Selection Medium

Kanamycin-containing medium at a level of 75 mg/l was used as an initial screen of the regenerated plants. Plants remaining green on this medium were grown on for further analysis i.e. Southern blotting and insect feeding trials or testing for virus resistance.

2) Trypsin assay

Assay Procedure.

To determine if the CpTi gene had been transferred to and was being expressed in the plant material, a trypsin assay was carried out. This assay examined transformed (and non-transformed) plant material for ability to reduce or prevent the enzyme trypsin from breaking down the substrate α -N-Benzoyl-DL-Arginine p-Nitroanilide (BAPNA), to release the product p-nitroaniline. The rate of hydrolysis of the substrate being indicated directly by the colour of p-nitroaniline released.

Trypsin assay procedure.

BAPNA solution

1 mg/ml α -N-Benzoyl-DL-Arginine p-Nitroanilide (BAPNA) in 0.1 M Tris buffer containing 0.04 M CaCl_2

Trypsin solution

10 mg in 0.0025 M HCL (pH 3.1)

Preparation of plant material for assay.

10 mg of plant tissue was placed into an eppendorf tube with 400 μl of 0.01 M NaOH and this was left for 1 hour, stirring occasionally. After 1 hour the contents of the tube were spun down for 5 mins. 100 μl samples were pipetted into the trypsin assay mixture.

Trypsin Assay

Trypsin activity was assayed by incubating 0.1 ml aliquots of the trypsin solution with 0.7 ml of BAPNA solution and 100 μl of plant extract. This was incubated for 0-60 mins at 37°C after which time the reaction was stopped by the addition of 125 μl 30% glacial acetic acid.

A negative control was prepared using non-transformed plant material and a positive control was prepared without acetic acid or plant material. The reaction tubes were spun for 5 mins to precipitate protein and the absorbance read at 410nm. Blanks of each

tube were prepared by adding acetic acid at time zero to identical tubes.

3) Southern Blot

As described in chapter 5.

4) Insect Feeding Trials.

Probably the best method of analysing transformed plants is by the use of insect feeding trials on transformed and control plants.

Although the plants detailed in this chapter are too small at this time for insect feeding, it is envisaged that within 1 year the plants will be of a suitable size from which to remove leaf tissue for insect feeding. Cultures of the appropriate pests are being established (S. Gordon, Zoology Department SCRI) for use in such trials.

5) Graft inoculations

Inoculations of transformed plants by grafting, will be carried out as the ultimate test in the usefulness of the AMV sequences for plant protection. This will be done with the help of the Virology Department at SCRI.

Experiments and Results.

Inoculation of explants with LBA4404 and 3 binary vectors.

Table 6.1. Total number of inoculations to date of explants with LBA4404:pROK/CpTi+5.

Sunberry	Inoculated	Control
No. leaf discs	400	400
No. died	301	27
No. callused	57	170
No. regenerated	62	203
No. plantlets produced	76	276
No. plants surviving	34	274

Table 6.2. Total number of inoculations to date with LBA 4404:pROK A3/3.

71WC64	Inoculated	Control
No. leaf discs	400	400
No. died	330	99
No. callused	43	122
No. regenerated	47	179
No. plants produced	49	260
No. plants surviving	23	236

Table 6.3a. Total number of inoculations to data with LBA4404:pROKCP+.

71WC64	Inoculated	Control
No. bases	40	40
No. died	32	9
No. regenerated	4	17
No. plants produced	4	22
No. plants surviving	2	0

Table 6.3b. Total number of inoculations to data with LBA4404:pROKCP+.

Glen Clova		
	Inoculated	Control
No. leaf discs	40	40
No. died	20	24
No. regenerated	7	16
No. plants produced	9	27
No. plants surviving	4	4

Table 6.3c. Total number of inoculations to data with LBA4404:pROKCP+.

Glen Moy		
	Inoculated	Control
No. leaf discs	40	40
No. died	40	4
No. regenerated	0	21
No. plants produced	0	29
No. plants surviving	0	26

The following figure (6.1) shows plantlets regenerating from base tissue of strawberry selection 71WC64 inoculated with LBA4404:pROK A3/3.

Fig. 6.1



Plantlets regenerating from base tissue of strawberry selection 71WC64 inoculated with LBA4404:pROK A3/3.

Analysis of regenerants.

Regenerated plantlets which looked healthy were assayed initially by placing into kanamycin medium as described previously.

In addition regenerated plants from inoculations involving the CpTi gene were assayed as described.

In total only 14 plantlets appeared healthy enough to withstand the effect of kanamycin and were placed onto antibiotic medium for 5 weeks.

Kanamycin Assay Results

Table 6.4. The effect of kanamycin on inoculated regenerated plantlets and non-inoculated controls.

	Regenerants from inoculated explants	Controls
No. plants	14	15
No. whitened	8	15
No. green	6	0

Trypsin Assay Results.

Table 6.5. Increased absorbance over time from a control, a non-transformed and an inoculated plant, increasing absorbance is directly related to the production of p-nitroaniline.

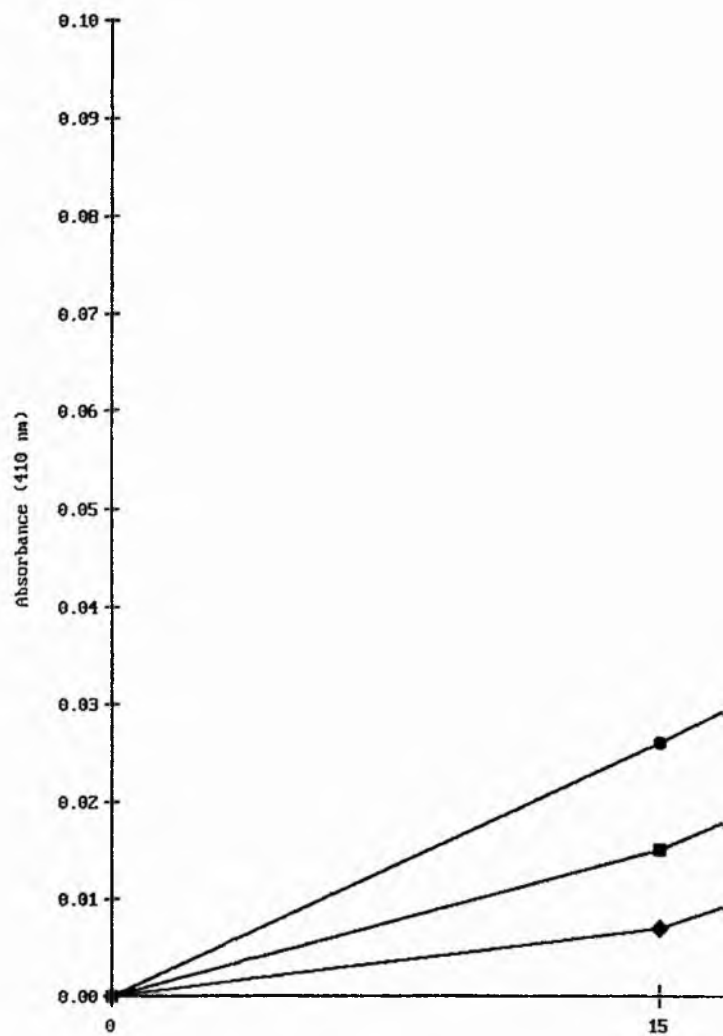
	Absorbance over time (mins)			
	0	15	30	60
Control	0	0.026	0.054	0.095
Non-transformed plant	0	0.015	0.039	0.066
Inoculated plant	0	0.007	0.026	0.049

Figure 6.2 on page 305 illustrates the activity of

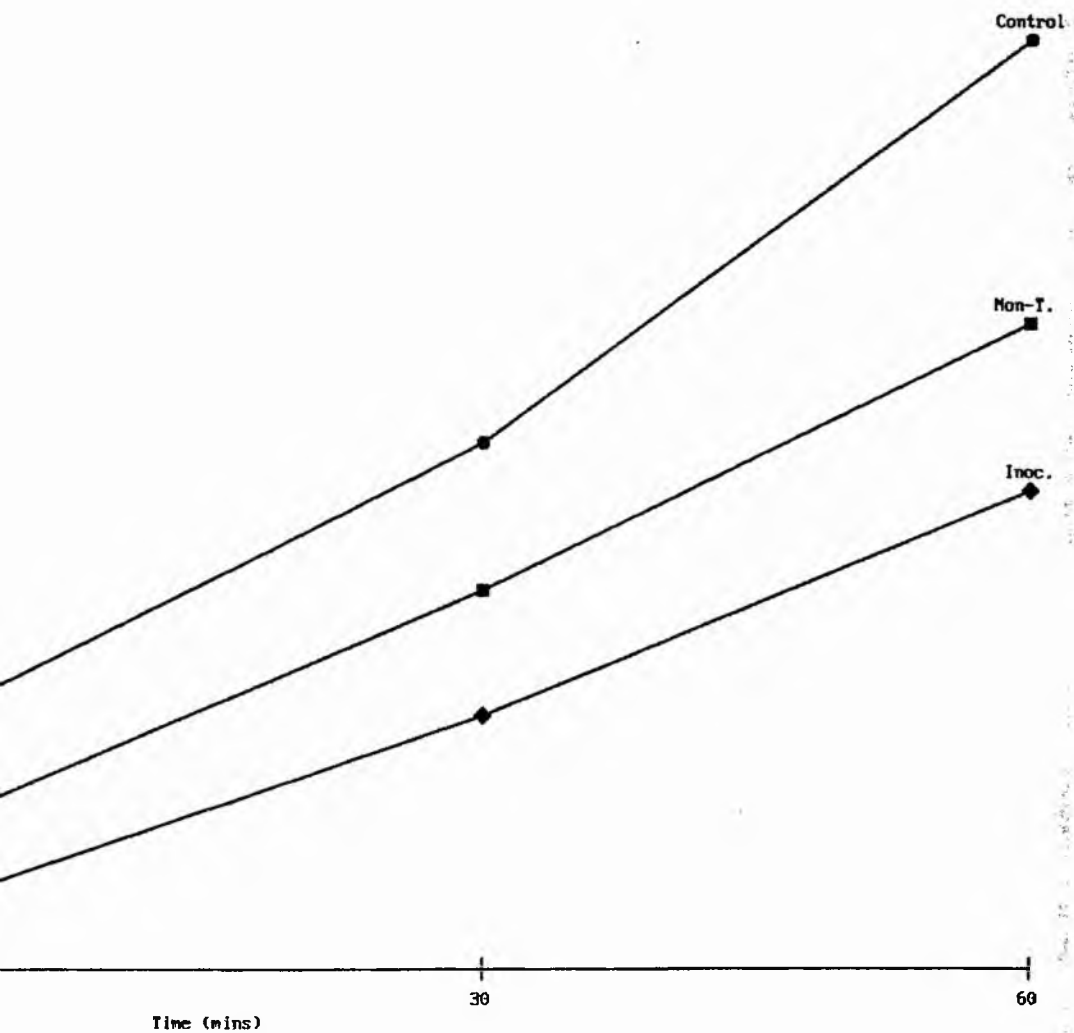
Trypsin on BAPNA as shown by an increase in absorbance due to the production of p-nitroaniline. Trypsin activity is reduced in the presence of plant extract for both control and inoculated plants, however a greater reduction is seen from the inoculated plant material.

Fig. 6.2

Figure 6.2 illustrates the increased absorbance over time due to the breakdown of BAPNA by the action of Trypsin to produce p-nitroaniline (Control). This reaction was also carried out in the presence of a non-inoculated plantlet (Non-T) and an inoculated plantlet (Inoc). A greater decrease in trypsin activity can be observed in the presence of the inoculated plantlet suggesting transformation has occurred.



Increased absorbance over time.



Discussion.

This chapter was concerned with research recently initiated using the developed transformation system, to introduce genes for specific improvements into soft fruit.

Inoculations of genotypes from Rubus and Fragaria spp. were carried out. Sunberry was inoculated with LBA4404:pROK/CpTi+5, selection 71WC64 was inoculated with LBA4404:pROK A3/3 and LBA4404:pROKCP+, and the red raspberries Glen Clova and Glen Moy inoculated with LBA4404:pROKCP+.

Of the 400 inoculated leaf discs from the cv. Sunberry, 62 regenerated, producing 76 plantlets, of which 34 survived and are being grown on for analysis. Selection 71WC64 regenerated 53 plantlets (49/400 from leaf discs and 4/40 from bases), of which 25 survived to be grown on for analysis. From the red raspberries 9 plantlets were produced from 40 explants of Glen clova, 4 surviving to be grown on further, however no plantlets were produced from Glen Moy. The regeneration of plantlets from explants in the above inoculation experiments (involving potentially useful genes) was slightly lower than those in chapter 5 (involving marker genes). In chapter 5, approximately 23% of inoculated explants of Rubus spp. regenerated plantlets compared with approximately 17% in this chapter, and from strawberry 16% of explants regenerated compared with 11% here.

Very little analysis has been carried out so far on the regenerated plantlets. As the plantlets have only recently been produced, their slow growth (mentioned in chpt.5) means that a long period of time is required before enough material is available for assay. From the findings in chapter five where the plantlets had only reached a size of 2 cm by 20 weeks of culture, it appears that the plants will require at least another 6-8 weeks before Southern blots can be carried out. Preliminary assays involving kanamycin selection and the assessment of trypsin inhibition from inoculated plant material have been carried out on a few of the healthier plantlets. Of the 63 plantlets produced only 14 were chosen for kanamycin assay. These included 9 Sunberry, 4 selection 71WC64 and 1 Glen Clova. One Sunberry was chosen to be analysed for the ability to reduce or inhibit trypsin.

Of the fourteen plants placed onto kanamycin containing medium, six remained green whereas the control plants all whitened on the same medium. Only one plantlet was analysed for trypsin activity, and the results from this assay suggested that some amount of inhibition had occurred.

Trypsin activity in the presence of non-transformed and inoculated plant extract was compared with a control assay involving only trypsin and BAPNA. The non-transformed plant exhibited around 70% Trypsin activity of that of the control, (some amount of reduction in Trypsin activity was expected, due to the addition of

plant extract changing the optimum conditions of the control assay) whereas the inoculated plant only exhibited 51% of the activity of the control. This is illustrated in figure 6.2 on page 305.

Although the kanamycin and trypsin assays appeared to yield some positive results, they were only preliminary assays. The regenerated plantlets have still to be analysed further, both by molecular techniques and bioassays, before any real conclusions can be made as to whether the genes have gone in and what level of resistance (if any) they will confer.

If the genes have been inserted into the plant material and confer resistance on the plant, field trialing and risk assessment procedures will be carried out (briefly discussed in chapter 7). Plant growth and other characteristics will also be assessed to determine any detrimental effects foreign gene insertion has on the plant.

The development of regeneration techniques, a transformation system and the subsequent availability of useful genes however means that in soft fruit the potential now exists for the specific improvement of successful cultivars without altering other characteristics.

Chapter 7

**The transformation system developed for soft fruit:
prospects and problems.**

**The transformation system developed for soft fruit:
prospects and problems.**

This thesis has been concerned with the development of a gene vector technique to be used as a tool in conjunction with conventional hybridisation techniques for the improvement of soft fruit genotypes.

The development of an appropriate system involved tackling a number of different problems. Initially the ability of the soil bacterium Agrobacterium to infect the cv. Tayberry was examined in order to determine its usefulness as a vector system. Agrobacterium naturally transfers a portion of its own DNA into some plant species upon infection, leading to crown gall or hairy root disease, useful markers of gene transfer. Four A. tumefaciens isolates and three A. rhizogenes isolates were examined for their ability to induce the symptoms (galls or hairy roots) of gene transfer and gene expression on Rubus species. Five of the isolates very successfully (80-100%) produced symptoms on the explants. From these and other inoculation experiments involving soft fruit two isolates were chosen, LBA4404 a disarmed Ach5 with resistance to the antibiotic rifamycin and Ar9402 wild type A. rhizogenes, also resistant to rifamycin, for use in the development of the system. The disarmed A. tumefaciens isolate was chosen to overcome the problems associated with expression of the hormone genes on the T-DNA, and

rifamycin-resistant isolates aided the identification of Agrobacterium containing a kanamycin-resistant binary vector.

The inoculation of whole plants with the Agrobacterium isolates described in chapter 2, although showing Agrobacterium to be capable of gall or hairy root production on wounded stem explants of soft fruit, did not lead to whole plant transformation, as only the cells at the site of infection contained the foreign DNA. The next step, therefore, required that regeneration systems be developed for small explants.

The requirement of regeneration systems from small explants were such that leaf discs or internodal segments could be inoculated and incubated with Agrobacterium to enable gene transfer into the cells to occur. The inoculated cells were subsequently induced to divide and form whole plantlets, some of which should have contained the foreign DNA in every cell.

Regeneration experiments were first conducted on Rubus spp., where regeneration was achieved on a simple medium NA, based on M+S salts with a few additions. The importance of medium composition, hormone combination, leaf orientation and light were examined in turn.

Interestingly, regeneration was also achieved for Fragaria, Ribes, and Vaccinium spp. on the same medium (NA) as for Rubus spp. provided the appropriate hormone combination for each species was included. No leaf disc system was developed for blackcurrants due to difficulties in sustaining survival of the explants.

Recently, however, progress has been made towards the development of a regeneration system, with successful callus production achieved from leaf discs which had not been placed under controlled fluorescent lights.

Once suitable regeneration techniques were developed, transformation experiments were initiated. Initially the only binary vector available for use in my experiments was Bin 19. This vector contains the NPT II gene useful as a selectable marker conferring kanamycin resistance to plants containing the gene. Although this antibiotic could not be added to the regeneration medium directly after inoculation, because of complete inhibition of regeneration, it could be used to select transformants once regeneration had been initiated. Due to the possibility that escapes (non-transformed plants which remained green) may have occurred on selection medium, dot blot and Southern blot assays were also carried out as part of the screen to identify transformants. The slow growth of inoculated regenerated plantlets however, meant that little material was available for the assays, which were therefore carried out on very small amounts of plant material (<1 g). The dot blot assay worked successfully with small amounts of material, but for Southern blots, isolation of DNA from such small amounts proved difficult. Problems were encountered in obtaining any DNA, and in cases where DNA was isolated, polyphenols also appeared to purify with the DNA. A recently published protocol by Couch and Fritz (1990) for the isolation of DNA from plants high in

polyphenolics may prove useful in future transformation experiments, assuming it can be carried out on less than 1 g of plant material.

The binary vector PBI121 containing the Beta-glucuronidase sequence proved very useful in the transformation experiments. This gene has a very simple and reliable assay which can be used to confidently identify transformants on 0.1 g amounts of plant material. The only drawback of the GUS construct used in my experiments was its expression in Agrobacterium. This meant that care had to be taken to ensure plant material destined for assay was free of Agrobacterium thus avoiding false positives. Differences in fluorescence, however, were observed between Agrobacterium expressing GUS and plants expressing GUS reducing the danger of false positives. The X-Gluc staining procedure (which results in a blue precipitate at the site of GUS enzyme activity in tissues) also allows GUS expression to be detected in the plant, which if only due to Agrobacterium contamination would be identified.

Recently an intron-containing GUS gene has been constructed (Vancanneyt et al. 1990) which is not expressed in Agrobacterium, prokaryotes having no splicing mechanism. This gene in addition to being useful in the identification of transformants, should help in the elucidation of early transformation events.

A construct with this intron-containing GUS gene is now being used in an attempt to transform blueberries. Blueberry transformation experiments have so far failed

to produce any regenerants, the leaf tissue dying a short time after inoculation. However this construct will allow any gene transfer which is occurring to be detected without the need for frequent antibiotic dips to remove Agrobacterium contamination (the dips possibly being an important factor in causing the death of leaf tissue).

The development of a transformation system in Rubus, Ribes and Fragaria spp. has lead to possibilities in plant improvement by the insertion of specific genes.

The transfer of inserted genes into the progeny has not yet been demonstrated in soft fruit, due to the very slow growth of the transformed plant and their lengthy life cycle. However, due to the findings in other crop species, experiments have been initiated to insert potentially useful genes into Rubus and Fragaria spp.

Investigation of transferred DNA carried out in tobacco have shown that foreign genes may pass through meiosis with a high degree of stability. The segregation ratio of the inserted gene in selfed progeny was close to a 3:1 ratio and segregation analysis of the following generation produced the expected 1:2:1 ratio of homozygous resistant, heterozygous resistant and sensitive plants (Muller et al. 1987). Transformants were also found to contain only one copy of transferred DNA per diploid genome (Muller et al. 1987).

A number of general observations have been made concerning transformation based on the findings of research conducted on a number of plant species

(Dunwell and Paul 1990): the copy number of DNA is between 1-50, there is generally one site of integration which appears to be random, and the expression and inheritance of inserted DNA is usually good. There are still a number of outstanding questions which have to be answered for all transformed plant species including: are there particular sites for integration, what determines the number of copies, is it possible to target gene insertion, how stable is expression over generations and does insertion of one gene effect that of another?

The transformation system developed for soft fruit has immediate implications for improving soft fruit cultivars, including the production of pest and disease resistant plants already briefly discussed.

The following section briefly discusses uses of the transformation system in soft fruit and other crops and finishes by outlining the steps which have to be taken before transformed plant material can be released into the environment and gain consumer acceptance.

Pest and Disease Control.

As discussed in chapter 6, immediate possibilities exist for disease and pest control by the insertion of foreign genes into soft fruit. Experiments have been initiated (Chpt.6) to introduce the Cowpea protease trypsin inhibitor gene (CpTi) into Rubus and Fragaria

spp. The CpTi gene has been inserted into tobacco (Hilder et al. 1990) where the majority of transformed plants were eaten by the pests, however a few plants were found to be resistant. Plants which showed the greatest expression of CpTi exhibited the highest insect mortality. Hilder et al. (1990) found a lack of correlation between the number of copies of the inserted gene and the amount of resistance. Gene expression occurred at different levels in all individually transformed plants.

Variation in gene expression was found in my experiments (exp.18, chap.5), where the amount of GUS expression was measured in a number of individually transformed plants. These results demonstrate the need for a second selection system to be applied after plants have been selected which show foreign gene expression. This second selection system is required to obtain plants with the most effective level of expression for the particular purpose of the inserted gene.

Hilder et al. (1990) also examined the effect of insertion of a second resistance gene, the pea lectin gene in tobacco. Plants produced generally had a normal appearance, however sometimes differences were visible in particular characteristics such as a reduction in dry weight, shape of leaf and internode length.

Aside from the CpTi gene, other available genes of potential value in soft fruit include the coat protein gene and the satellite gene of arabis mosaic virus

(AMV). Again these have to be selected for not only by Southern blotting, but also by the use of bioassays.

Unlike the CpTi gene, high levels of expression of the tomato mosaic virus coat protein gene did not give the most effective control of the virus in tomato and potato. In this case a lower expressing clone exhibited the greater resistance (Reavy 1990). Again selection has been carried out on all transformed plants to find those which are most resistant.

Another potentially useful viral gene in raspberries is the coat protein (CP) of the pollen borne raspberry bushy dwarf virus (RBDV). This virus, briefly described in chapter 6, can be a serious problem in cvs. such as Glen Moy and Glen Clova. The CP gene of RBDV has recently been isolated and cloned (Mayo pers. commn. 1990) into a Bluescript plasmid (Short et al. 1988). Work is now underway to insert this gene into an Agrobacterium binary vector for use in the transformation of the above cultivars (Graham, Mayo and McNicol 1990).

Manipulation of Qualitative Traits.

Apart from the introduction of genes to control pests and diseases, genes can be introduced for their effects on qualitative characteristics. The introduction into petunia of a maize dihydroflavonol reductase enzyme resulted in synthesis of pelargonidin producing brick-red flowers (Meyer et al. 1987). Petunia hybrida is

unable to convert kaempferol into pelargonidin due to the specificity of the biosynthetic enzyme dihydroflavonol-4-reductase (DFR), but the maize enzyme is capable of this. In blackcurrants where juice is of great economic importance and juice quality is one of the main breeding objectives, manipulation of pigment content could prove very useful.

Study of Gene Function and Gene Regulation.

Transformation systems as well as being of use for the insertion of new characteristics into plant species can be used in the study of gene function and the mechanisms of gene regulation. The identification of cis-acting elements responsible for the development and tissue-specific regulation of transcription can be elucidated by fusing various lengths of 5'-upstream regions of the genes to marker genes which are subsequently integrated into plant genomes using Agrobacterium.

Bogue et al. (1990) studied seed proteins which are highly regulated with mRNA accumulating rapidly during the maturation phase of embryogenesis, and fused a fragment containing the upstream region of the sunflower helianthinin seed storage protein to the GUS reporter gene. GUS expression driven by the helianthinin upstream region was developmentally regulated in seeds. Expression of the sunflower helianthinin seed storage protein in tobacco was also developmentally regulated.

Shewry et al. (1990) are currently studying the structure and control of gene expression of three families of genes that determine cereal seed quality. In many species the nutritional value of seed proteins could be significantly improved by raising the concentration of particular amino acids essential in human and animal diet. Researchers are examining what constitutes quality which can subsequently be altered once transformation has been effected. The 5'-upstream regions of the genes have been fused to the coding regions of marker genes and inserted into the tobacco genome using Agrobacterium. Studies on gene controlling sequences and on analysis of the storage proteins encoded by the genes should lead to improved cereal quality by genetic engineering once transformation of cereals has been effected.

Antisense its Role in the Inhibition of Gene Expression.

Alteration of biochemical pathways to reduce or irradiate undesirable traits or change developmental programmes can also be accomplished by Agrobacterium transformation, using antisense RNA. Antisense RNA can be used to inhibit translation. Antisense RNA complementary to a specific mRNA hybridises in vivo, disrupting the normal processing of the mRNA. The ability of antisense to inhibit transient expression of the chloramphenicol acetyltransferase gene was demonstrated in carrot protoplasts by Ecker and Davis

(1986). More recently it has been shown to repress expression of the nopaline synthase enzyme (Rothstein et al. 1987 and Sandler et al. 1988) and the CAT enzyme (Delauney et al. 1988) in transgenic tobacco plants.

Antisense RNA has also been used to alter the levels of the polygalacturonase (PG) enzyme in tomato. Grierson et al. (1990) have produced transgenic tomato plants with 1% the normal levels of PG.

Polygalacturonase is involved in the structural changes which occur in the cell wall and lead to fruit softening (Hobson 1964, Hobson 1965 and Brady et al. 1982). Alteration of the levels of this enzyme could be used to overcome fruit deterioration. By examining a number of individually transformed plants it should be possible to select one with a desirable level of expression to overcome or reduce fruit rot.

This would also enable studies to be carried out on the role of PG during ripening, as inhibition of translation of a particular genetic sequence may result in phenotypic changes revealing information on the role of the gene.

Antisense RNA has also been shown to downregulate an enzyme specific to lignin biosynthesis, cinnamyl alcohol dehydrogenase (CAD) (Schuch et al. 1990) and inhibition of flower pigmentation by antisense to the petunia chalcone synthase (CHS) gene (Krol et al. 1988) has been demonstrated. Substrates of the CHS enzyme are colourless thus reducing the amount of CHS enzymatic activity leads to fainter colours of even white flowers.

As well as antisense genes, sense genes surprisingly have been shown to reduce or inhibit expression of the CHS gene, giving rise to pure white flowers (Mol et al. 1989).

There have been reports suggesting that a large excess of antisense RNA is required for effective inhibition (Ecker and Davis 1986) and others where a large excess is not required (Cannon et al. 1990). Suggested mechanisms for antisense RNA action include: (i) the prevention of ribosome binding by duplex RNA, (ii) the RNA duplex is not transferred into cytoplasm (Kim and Wold 1985), and (iii) the RNA duplex is highly unstable and so degraded in the nucleus (Crowley et al. 1985).

Antisense RNA has been suggested as a method of plant protection against viruses, but its usefulness has yet to be determined. Antisense RNA is produced in the nucleus, whereas generally the virus exists in the cytoplasm, and therefore the chance of meeting may be remote. Usefulness may therefore be limited to those viruses having a nuclear phase.

Ribozymes

Ribozymes may be of more use against viruses than antisense RNA. Ribozymes are catalytic RNA molecules with the ability to cleave RNA in a specific way, thus inactivating it (Haseloff and Gerlach 1988). Through the use of Agrobacterium transformation it should be

possible to introduce and express ribozyme sequences specific to any RNA substrate desired.

Transposons and Gene Isolation.

Agrobacterium as well as inserting useful genes into plants also has a role in the isolation of other genes. Isolation and characterisation of transposable elements in maize has led to the use of cloned transposons (Tn) as probes for the detection of mutated plant genes (Fedoroff et al. 1984, Motto et al. 1988, O'Reilly et al. 1985). It has been shown that the maize Ac element can transpose when inserted into other species such as Nicotiana tabacum (Baker et al. 1986), Daucus carota, Arabidopsis thaliana (Van Sluys et al. 1987) and Solanum tuberosum (Yoder et al. 1988).

The activity of transposable elements (Fedoroff 1984) can be detected if the Tn integrates into a gene inducing a mutation. If the gene has a role in determining phenotype, then visual differences will occur. For example, if a gene involved in pigmentation is affected by transposon mutagenesis, a recognisable change in phenotype is produced. Genetic analysis of transposition has been aided by the use of a phenotypic assay allowing direct selection of transposition excision events. When Ac transposes from the leader sequence of the Neomycin phosphotransferase II gene, expression is restored (Baker et al. 1987).

Work has been initiated using the developed transformation system to isolate a spinelessness gene (Sfw) from the diploid raspberry cv. Willamette by introducing the Ac transposable element of maize (Graham Kumar and McNicol 1990). This gene, if isolated, would be of use in cultivars such as Tayberry, Sunberry and Leo. Initially, a transposon has been inserted into Sunberry plants and excision of the 4 Kb Ac transposon from between the promoter and coding sequence of NPT II gene is being examined.

Transformed Plants as Minature Factories.

The techniques developed for the improvement of crop species, have lead to the possibility of transforming tobacco plants to express useful human proteins. Oncogene, a Seattle based biotechnology company recently reported that a protein from the leaves of pokeweed:- pokeweed antiviral protein, may prevent cells becoming infected with HIV. The Agricultural Genetics Company (AGC) have successfully cloned this gene and are looking at the possibility of inserting this gene along with an antibody that targets specific cells of the human immune system into a "factory" plant such as tobacco.

Other companies such as Monsanto plan to market products grown in tobacco plants. One such product is a sunscreen containing human melanin, a natural skin pigment grown in tobacco.

Transgenic Foodstuff in the Market Place.

Although transformation is at an early stage in soft fruit, in a number of cases the use of inserted genes for herbicide resistance and insect resistance is close to practical exploitation.

The Bacillus thuringiensis (Bt) toxin gene has been shown to confer insect resistance in transgenic tobacco and tomato plants against the tobacco hornworm (Vaeck et al. 1987) and tomato pinworm (Fischholf et al. 1987). In the transgenic tomato plants, despite the low level of RNA expression, the insecticidal protein was produced at a sufficient level for control. Differences in the amount of expression from plant to plant were seen through the results of bioassay. Spore preparations of this bacterium have been used as a biological insecticide for some 20 years, though the high production costs and instability of the crystal protein in the field has limited their use. The use of transgenic plants producing the toxin should overcome these problems, and, due to their toxicity being highly specific, they should provide a new and environmentally safe insect control method.

Herbicide resistance genes have been inserted into a number of plant species. Tomato (Fillati et al. 1987), soybean (Hinchee et al. 1988) and rape plants (Fry et al. 1987) with resistance to glyphosate and cotton resistant to 2,4-D (Llewellyn 1990) have been produced.

Possibly within the next 5-10 years, transgenic plants or their products will reach the agricultural market place. This assumes that extensive field trials have yielded positive results and risk assessment procedures have been carried out.

Small scale field trials have been carried out in the USA, Canada and Europe, mainly on material resistant to herbicides due to the availability of these resistance genes. The production of herbicide-resistant plants causes concern, due to the possibility of encouraging greater dependence on toxic chemicals. Some groups have taken a very critical stance over the production of herbicide resistant crops (Anon. 1990). Industry, producing these plants, however, argue that the environment and the farmer will benefit from the use of low toxic very specific herbicides.

The wide scale trialing of transgenic plants may be hampered by the inconsistent regulatory requirements applied throughout the world.

Aside from field trials to assess the impact of a foreign gene on plant characteristics, risk assessment procedures have to be carried out into the safety of the inserted gene. At SCRI risk assessment procedures in the form of gene flow from the institute are being carried out. Prof. J. Luby, a visiting scientist is currently looking at gene flow of a spinelessness gene, which originated at SCRI into wild raspberry populations. This study will indicate how likely it is that foreign genes

introduced into specific cultivars will integrate into wild soft fruit populations around the institute.

Another type of risk assessment procedure, in the form of toxicity tests, has to be carried out for each gene inserted. For the CpTi gene, toxicity tests have been carried out on mice. This gene should provide a safe and acceptable method of insect control since it occurs naturally in food crops which can safely be eaten raw. As the CpTi inhibits an insect digestive enzyme, resistance to the CpTi by insects would require a reorganisation of digestive physiology.

In assessing the risk of using virus sequences, other procedures should be carried out. There are a number of possible risks in the use of virus sequences for disease control. With coat protein (CP) genes the possibility exists that the coat protein of one virus could wrap around another (transcapsidation) spreading the virus to new hosts. This can be induced to happen if the CP of Cucumber mosaic virus (CuMV) is mixed with the nucleic acid of tobacco mosaic virus (TMV), but it remains unknown whether or not this happens in plants, which in nature can be infected with more than one virus at a time (Hull 1990). As mentioned in chapter 6, viral satellites can also enhance as well as reduce or eliminate the symptoms of disease and could therefore potentially be very destructive. It seems unlikely that satellite protection would gain approval unless engineered satellites which could not enhance symptom production were used.

Once transgenic plant material has been assessed and is at a stage for large scale release into the environment, consumer opinion (and how to promote transgenic foodstuff to the consumer) must be considered. In the USA, initial public resistance to transgenic foodstuff has largely disappeared. To date in the U.K., the public seem largely unconcerned about how the improvements are made and more concerned with the benefits obtained. The Channel 4 consumer programme "Check Mate" (13/11/1990) suggested that any controversy which does exist over transgenic foodstuff is due mainly to ignorance. It suggested secrecy by scientists over developments in biotechnology leads to fear and suspicion. For the first time, this year, MAFF released a statement to the press on the availability and use of a genetically engineered yeast. This will hopefully lead to a wider awareness, and therefore acceptance, of transgenic foodstuff by the general public. "The Courier and Advertiser" a local Dundee newspaper, has published only one article on genetic engineering in the last 2 years. This article (published 19/4/1990) dealt positively with plant transformation, viewing it as one of the few ways of overcoming food shortage caused by a rapidly expanding population. Other reports from national newspapers have also viewed plant transformation favourably. "The Times" have published 3 reports of plant transformation so far in 1990 (17/3/1990, 29/3/1990 and 29/8/1990), two of which were favourable, discussing the possibilities of plant

transformation, and the third discussed the views of the Green party in West Germany to transgenic field tests.

The CpTi gene should be readily acceptable to the consumer since the transferred genes occur naturally in the edible parts of crop species. The CpTi gene, a naturally occurring method of plant protection would facilitate a reduction in the amount of harmful chemicals involved in production. The benefits of transgenic tomatoes, which do not rot, are easily seen, as are better looking virus and pest resistant plants producing higher yields at cheaper costs. Its more difficult to see how plants resistant to herbicides will benefit the consumer, and their use may be unacceptable.

Transgenic plants could be best presented to the general public as being produced by a process which occurs naturally anyway. The process being manipulated to introduce only one well characterised gene into a chosen plant whereas plant breeding, the traditionally accepted method of crop improvement (whose products have been consumed for centuries) transfers many genes.

In time, plant transformation is likely to become a routine technique for inserting genes of value into widely grown cultivars for specific improvements.

The significance of the development of these systems and the production of transgenic material, is that it may lead to the use of more environmentally safe herbicides, will permit a reduction of chemical input into the food chain (by reducing chemicals such as

insecticides and fertilisers) leading to increased quality and quantity of harvest.

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Appendix.

Abbreviations

α -N-Benzoyl-D-L-Arginine p-Nitroanilide	BAPNA
Beta-glucuronidase	GUS
6-Benzylaminopurine	BAP
Blackcurrant medium	BM
5-Bromo-4-chloro-3-indolyl glucuronide	X-Gluc
Chloramphenicol acetyltransferase	cat
2,4-Dichlorophenoxyacetic acid	2,4-D
6- oxo Dimethylallylaminopurine	DMAAP
Ethylene diamine tetraacetic acid	EDTA
Gibberellic acid	GA
Indole-3-butyric acid	IBA
Kanamycin	Kan
Kinetin	K
Luria-Bertani broth	LB
Murashige and Skoog medium	M+S
4-Methylumbelliferyl-B-D-glucuronide	MUG
4-Methylumbelliferone	MU
Metric tonnes	MT
Neomycin phosphotransferase type II	NPT II
Nopaline synthase	nos
Raspberry multiplication medium	RM
Rifamycin	Rif
Woody plant medium	WPM
Yeast mannitol broth	YM
Zeatin riboside	ZR

Chemical Information.

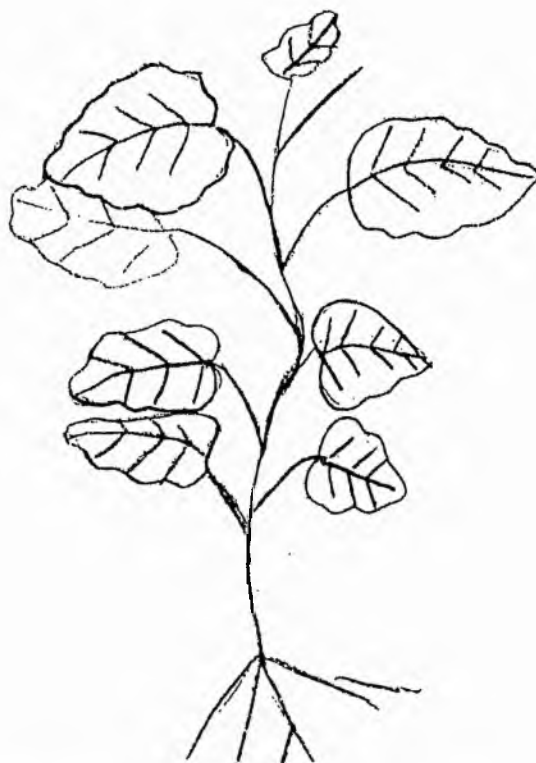
All chemicals were Analar grade obtained from Sigma unless listed below.

Chemical	Company	Catalogue number
Activated charcoal	Sigma	C6289
Agar	Flow Laboratories	26-661-17
Bactotryptone	Difco	0123-02-0
Benzylaminopurine	Sigma	B6750
Carbenicillin	Sigma	C1389
Cefotaxime	Rousell	claforan
2,4-D	Sigma	D8407
DMAAP	Sigma	D0636
Ethidium bromide	Sigma	E1510
Gibberellic acid	Sigma	G7645
Indole-butyric acid	Sigma	I5386
Kanamycin sulphate	Sigma	K4000
Kinetin	Sigma	K0753
Lysozyme	Sigma	L6876
M+S medium	Flow	26-100-20
Rifamycin sv.	Sigma	R8626
WPM salt solution	Sigma	M0154
WPM micronutrients	Sigma	M9913
X-Gluc	Sigma	B0522
Zeatin riboside	Sigma	Z0375

Tissue culture growth of four soft fruit spp.

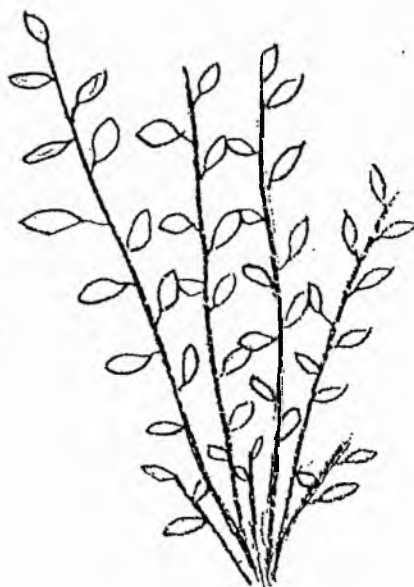
The following drawings (D1-D4) illustrate the growth in tissue culture of raspberry (D1), blueberry (D2), blackcurrant (D3) and strawberry (D4). The stock tissue cultures were initiated by introducing buds into tissue culture. These buds form whole plants within approximately 4 weeks after which they could be divided and used to initiate the stock culture. The raspberry and blueberry originate as single shoots, whereas the strawberry and blackcurrant grow as small groups of plants originating on tissue which forms at the base of the plant in a tissue culture environment (referred to as base tissue). Micropropagation of the raspberry and blueberry is carried out by cutting the single shoot produced into sections, each with an axillary bud and placing on fresh micropropagation medium. The blackcurrant and strawberry are divided into individual plants by cutting the base tissue into small sections and again placed onto fresh medium.

D1



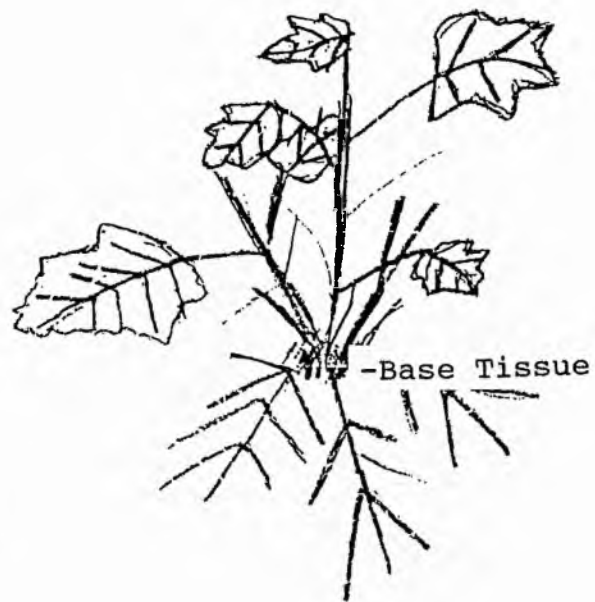
Tissue culture growth of raspberry.

D2



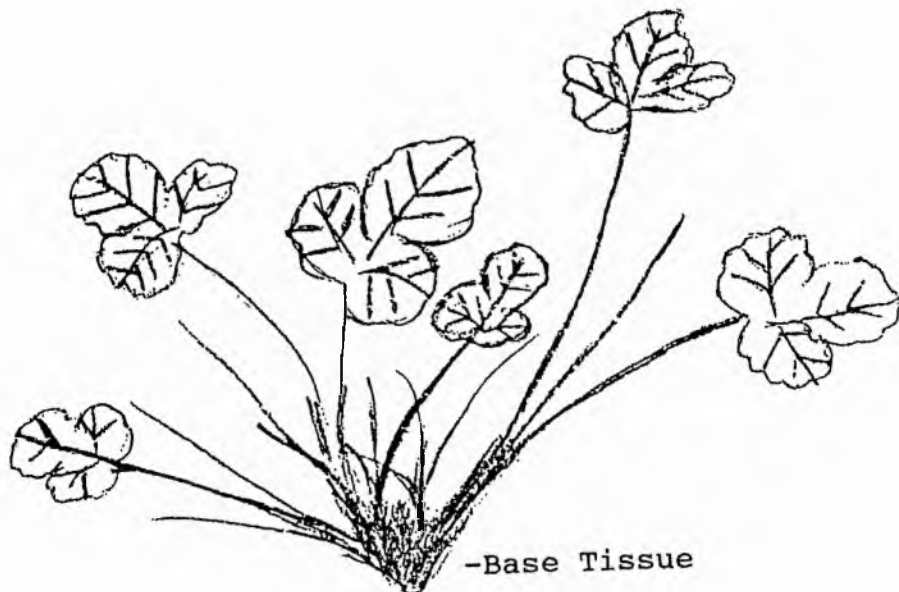
Tissue culture growth of blueberry.

D3



Tissue culture growth of blackcurrants.

D4

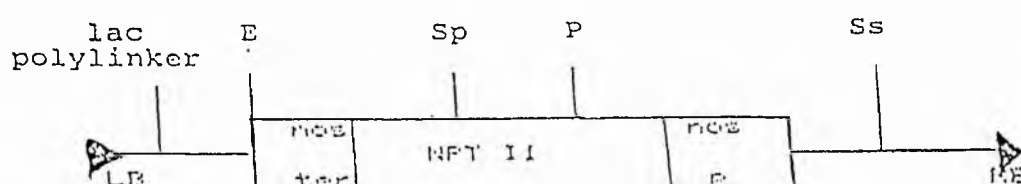


Tissue culture growth of strawberry.

T-DNA regions of 5 the binary vectors.

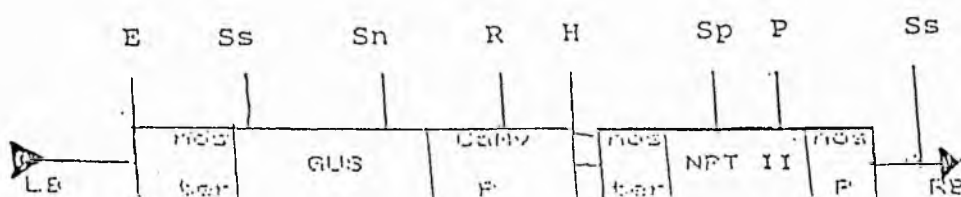
The T-DNA regions of the five binary vector constructs used in chapters 5 and 6 are shown below. All the constructs are based on Bin 19 and therefore contain the NPT II marker gene along with the specific gene of interest inserted with the control sequences into the polylinker.

Bin 19



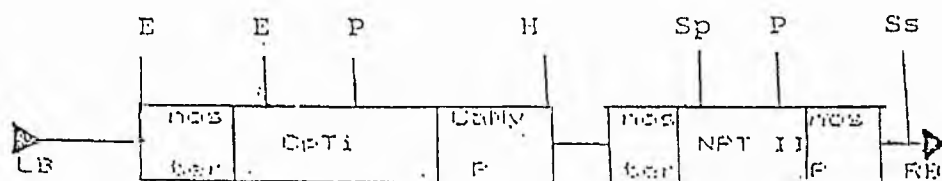
Ref: Bin 19 (Bevan 1984), was kindly supplied by Dr. D. M. Lewis, J.I.I., Norwich.

PBI121



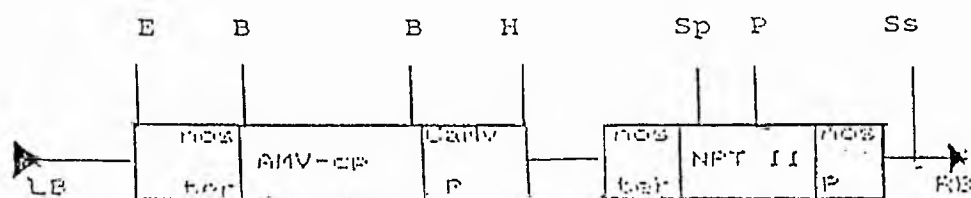
Ref: PBI121 (Jefferson 1987a,b) was kindly supplied by Dr. D. James, I.H.R., East Malling.

pROKCpTi+5

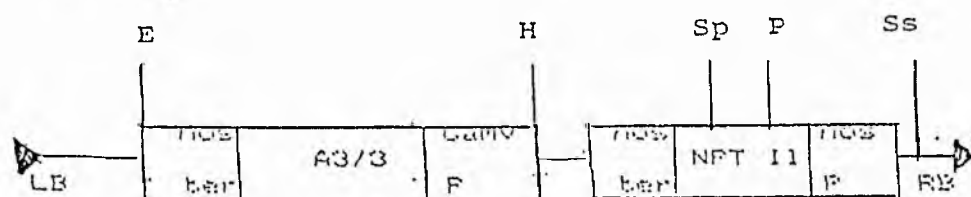


pROKCpTi+5 is on licence from A.G.C. Ltd.

pROKCp+



pROKA3/3



pROKCp+ and pROKA3/3 were kindly supplied by Prof. I. Cooper, Institute of Virology, Oxford.

Abbreviations:

B, Bam HI; E, Eco RI; H, Hind III; P, Pst I; R, Eco RV; S, Sna BI; Sp, Sph I; Ss, Sst II; LB, RB, left and right T-DNA border repeats; nos, nopaline synthase; p, promoter sequence; ter, terminator sequence; CaMV p, Cauliflower mosaic virus 35s promoter;